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Intersectin protein regulation by microRNAs in HEK cells and human neuronal stem cells

Master's thesis

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Tiivistelmä – Referat – Abstract

Intersectins (ITSNs) are important scaffold and adaptor proteins that play an important role in various cellular processes such as endocytosis. Although we know a lot about their function, there is little information on the regulation of these proteins. On the other hand, microRNAs have been shown to have an extensive function in regulating numerous genes in animals and their dysfunction is credited for down regulation of many proteins. In this study, I demonstrate that microRNAs are potential regulators of ITSNs in HEK293 cells and human neuronal cell cultures.

In this study, I cloned 3'UTRs of different isoforms of intersectins (ITSNs) and microRNAs to the expression vectors to express them in cells. I then transfected HEK293T or neuronal stem cell line (HEL47.2) with the constructed vectors and used various methods to analyse the effect of microRNAs on the expression of ITSNs. The main methods I used were dual-luciferase assay, reverse transcription quantitative PCR and western blotting, human neuronal stem cell culturing and lentiviral transduction.

My results demonstrate that there were two microRNAs that stood out from other and had a significant downregulation of ITSNs mRNA levels in HEK293T cells. Those were miR-124 and miR-19. However, in the human neuronal cell line I did not observe a significant alteration of the ITSNs transcript level. Additionally, I suggest that the given microRNAs regulate protein levels by promoting the decay of the ITSN transcripts. However, more studies are needed to show a stronger causative effect of microRNAs on ITSNs. Subsequent studies should also look at how multiple microRNAs can influence gene expression cooperatively.

Avainsanat – Nyckelord – Keywords
Instersectins (ITSNs), microRNAs (miRs), endocytosis, 3' untranslated regions (3'UTR)

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Muita tietoja – Övriga uppgifter – Additional information

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List of Abbrevations

3’UTR – 3’ untranslated region

AD – Alzheimer’s disease

Amp – ampicillin

AP2 – adaptor protein 2

DLA – Dual-luciferase assay

DNA – deoxyribonucleic acid

DS – Down syndrome

GTP – guanosine triphosphate

HEK293T – human embryonic kidney 293 cell line

ITSN1-L – intersectin 1 long

ITSN1-S – intersectin 1 small

ITSN2-L – intersectin 2 long

ITSN2-S – intersectin 2 small

kDa – kilo dalton

LB – lysogeny broth

miR – micro ribonucleic acid

mRNA – messenger ribonucleic acid

NSC – neuronal stem cell

qPCR – quantitative (real-time) polymerase chain reaction

RNA – ribonucleic acid

RT – room temperature

SV – synaptic vesicles

Introduction

For cells and tissues to function properly and carry out their tasks, they need to interact and communicate with one another by sending different kinds of signals to each other. In biological systems, the main way of communicating with one another is by sending chemical molecules from one cell to activate certain responses in other cells. Often, for cells to respond to the chemical signal, a given molecular cargo needs to be taken inside the cell (internalized) by a widespread and fundamental cellular process in eukaryotes, called endocytosis. In the most general form, the molecular cargo that is needed to be taken into the cell first interacts with cellular surface proteins. Then, the membrane buds inward and wraps around the cargo until the vesicle that is formed around it loses contact with the cell membrane and travels to its destination inside the cell. There are many endocytic pathways that vary in the formation, protein content and the structure of the vesicle. Currently, endocytosis as a process is divided into three major categories: clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin-independent endocytosis. In each type of endocytosis, after the uptake of the cargo, the endosomes are merged into common early endosomes. From there they can be further transported back to cell surface, to Golgi complex or across other compartments of the cell (Elkin, 2017). In this study, I will focus more on a clathrin-mediated endocytosis.

Clathrin-dependant/-mediated endocytosis is known to exist in all described eukaryotic organisms and was likely present in the last common ancestor of eukaryotes, more than billion years ago (Wideman, Leung, Field, & Dacks, 2014). One of the first reports of endocytic pathways is known from 1960s paper from Rosenbluth and Wissing about the uptake of intact ferritin proteins by toad spinal ganglion neurons. With a series of electron microscopy images, the authors were able to demonstrate that entire ferritin proteins were internalized by neurons. At the time, it was very surprising that neurons in general conduct such function. Authors described these small bodies as „coated“ vesicles and called the process a pinocytosis (1964). Nowadays, it is known as endocytosis and pinocytosis, often described as macropinocytosis, is now defined as a cellular uptake of bulk solutes without passing the cell membrane (Doherty & McMahon, 2009).

Intersectin or ITSN protein is known to be an important mediator in the process of endocytosis. It is a short scaffold and adaptor protein and its main role there is to initiate endocytosis by recruiting other proteins to the clathrin-mediated endocytic machinery. Lack of this protein or overexpression could potentially lead to the significant disruption of endocytosis which in turn

could lead to physiological stress. Regulation of gene expression by microRNAs is also known to play important part in human physiology. Moreover, many miRs are identified to function in brain where they target a variety of proteins such as BDNF, MAPT and LRRK2 (Sharma & Lu, 2018). The aim of my thesis was to study the regulation of ITSN proteins and its isoforms by microRNAs. In this work I used HEK293T and human neuronal cells HEL47.2 to express a variety of microRNAs to observe the expression levels of ITSN genes.

1. Literature review

1.1. Mechanism of clathrin-mediated endocytosis

Though the endocytosis as a process itself is conceptually fairly simple, there are still aspects that need deeper look. Over many decades, more than 50 different cytosolic proteins have been found to be part of endocytic machinery. Every protein in this process have its own certain function so that the whole pathway can run in a highly ordered manner (Kaksonen & Roux, 2018).

Clathrin protein is a central component in a clathrin-dependant endocytosis. The protein is made of a heavy chain (about 190 kDa) and a light chain (about 25 kDa). The heavy chain is made of three triskelions – outward protruding structures that resemble legs (Fig. 1.1). Triskelion structure helps clathrin to work as a structural backbone for clathrin-coated endosomes. All three legs of the clathrin interact with other triskelias and ultimately form a circular polygonal lattice around the vesicles (Kaksonen & Roux, 2018).

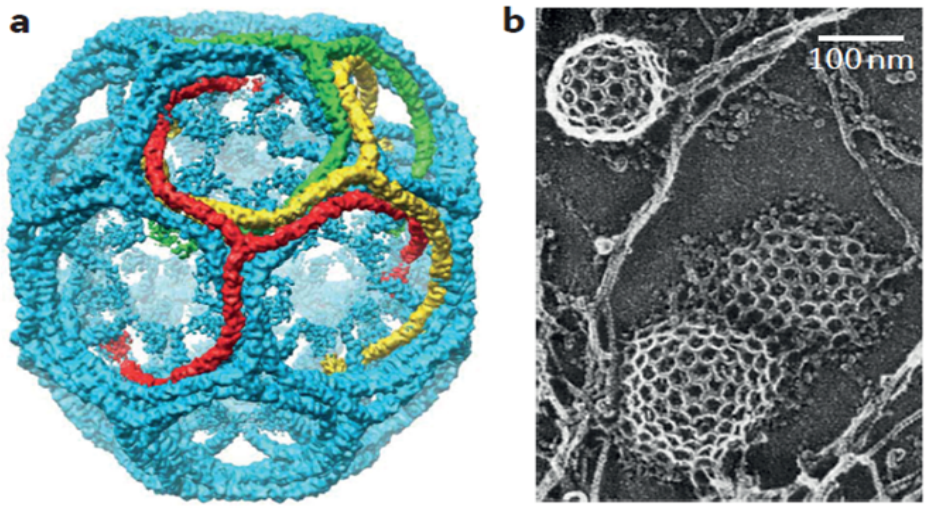


Figure 1.1. In image **A,** a structure of clathrin coated vesicle that is obtained by cryo-electron microscopy is shown. Red, yellow and green colours highlight individual clathrin proteins. Clathrin molecule (colored in red) have three triskelias that extend out. Both yellow and red clathrins interact with each other by aligning their heavy chains. Image **B** was obtained by electron microscopy and shows multiple lattices of clathrin-coated vesicle on the surface of fibroblasts (Kaksonen & Roux, 2018).

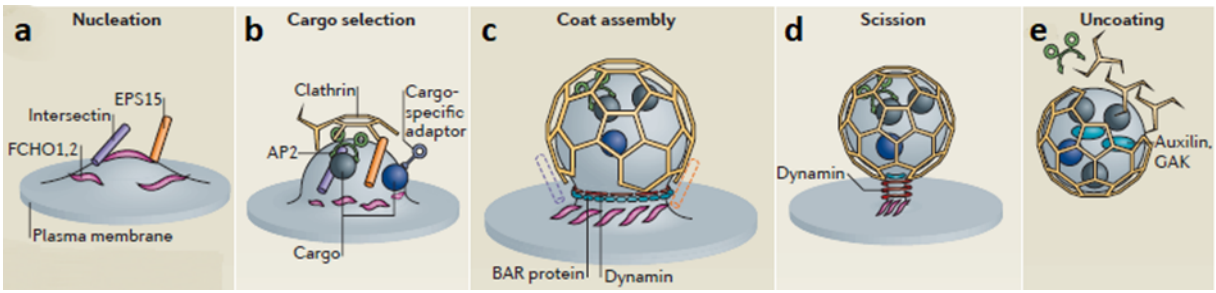
Clathrin-coated endosomes go through many morphological stages from the initiation to the uncoating of clathrin lattice. Based on the structural studies, the whole process has been divided into five separate stages. The formation of the endosome starts with the **nucleation** (McMahon & Boucrot, 2011). At first, the coating proteins need to assemble to the membrane. The initial budding module is formed by the following scaffolding proteins: FCH domain only proteins (FCHO), EPS15, epsin protein and lastly intersectin (ITSN) proteins (Kaksonen & Roux, 2018). These proteins bind specifically to the phosphoinositides (PIP_2) in the lipid bilayer which facilitate the assemble of vesicle forming proteins (Kaksonen & Roux, 2018).

The next step in this process is a **cargo selection**. Proteins in an early budding endosome are thought to bind adaptor protein 2 (AP2) at this stage. AP2 protein binds to the membrane receptors that are bound to certain cargo molecules. AP2 is the second most abundant protein in clathrin-mediated endosomes after clathrin. AP2 is a heterotetrameric adaptor protein which has multiple isoforms with combinations of different subunits (α, β2, μ2, σ2). AP2 binds to the cargo-specific adaptor protein thus mediating selection of molecular cargos. Currently, numerous receptor binding accessory proteins present in different cell types are known, which bind to different receptors and all of these cargo-specific proteins always bind to core adaptor AP2. Thus, it is also thought that the cargo selection could start at the same time as a nucleation stage (McMahon & Boucrot, 2011). Studies with AP2 knockout models have observed that

these vesicles also lack clathrin proteins. This, in turn, suggest the interaction between AP2 and clathrin (Motley, Bright, Seaman, & Robinson, 2003).

After recruitment of all the proteins to the site of endocytosis **clathrin coat assemble** occurs**.** Clathrin molecules polymerize and start to form the lattice-like structure around the vesicle. As the polymerization expands, the forming vesicle also invaginates more inward into the cell leading to the generation of a mature clathrin-mediated vesicle (McMahon & Boucrot, 2011). The bending of the membrane with the subsequent elongation of the invagination requires force production to overcome mechanical barriers – membrane tension and turgor pressure. Although the exact mechanism is still not known entirely, there are numerous theoretical models and chemical pathways through which cells can overcome membrane tension. According to one hypothesis, actin network around the site of endocytosis is responsible for generating membrane deformation. It is thought to be achieved through fast actin polymerization and depolymerization. But overall this question is still far from resolved (Lacy, 2018).

The release of the clathrin-coated vesicle or the **vesicle scission** depends on the GTPase dynamin. Dynamin is recruited to the complex by BAR-domain proteins to which it binds. Dynamin is a mechanochemical enzyme that uses energy from GTP hydrolysis to remodel membrane. Dynamins polymerize into a cylindrical scaffold at the neck of the endosome vesicles. From there the hydrolysis of the GTP generates a fission between the membrane and vesicle ultimately causing it to detach from the membrane (Bashkirov et al., 2009). Once the endosome is detached, clathrin coated lattice will also start to be **disassembled** back to individual clathrin proteins. It is catalysed by ATPase heat shock cognate 70 (HSC70) which is recruited by its cofactor auxilin. This allows uncoated vesicle to travel and fuse with its target destination – early endosomes. Meanwhile, the disassembled clathrins will be recycled again to form new endosomes (McMahon & Boucrot, 2011).



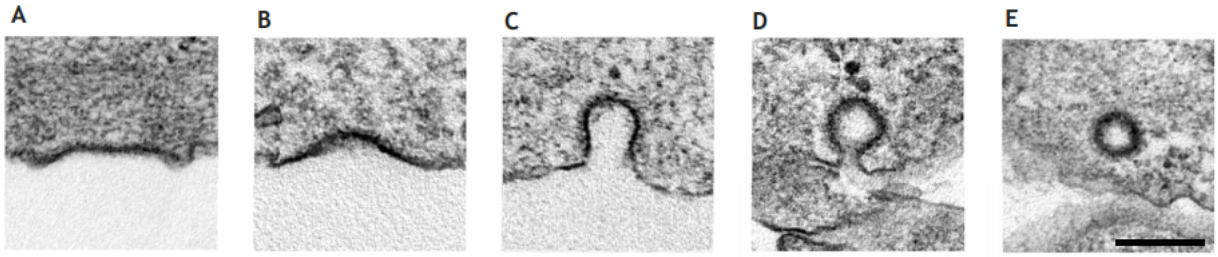


Figure 1.2. In the upper panel: The proposed five steps of clathrin-mediated endocytosis starting from nucleation (A), followed by cargo selection (B), coat assembly (C), scission (D) and lastly, uncoating (E). In the lower panel, images obtained by electron microscopy are shown demonstrating the corresponding steps *in vivo.* Scale bar: 200 nm. (McMahon & Boucrot, 2011; Haucke & Kozlov, 2018).

1.2. Endocytosis in neurons

The basics of the endocytic process is similar in neurons as it is in other cell types. However, there are some special properties in order to meet the needs of the neurons, especially, in the cargo specificity and the speed of the process in cases of a rapid and repeated firing between pre- and postsynaptic neurons (Saheki & Camilli, 2012). Endosomal trafficking has important roles in numerous aspects of neuronal processes, particularly in cell survival, axonal growth and guidance, dendritic branching and cell migration. Interestingly, in neuronal signal transduction, cellular or physiological responses can vary based on the cellular location of the signal initiation. The most widely studied endosomal signalling pathways in neurons involves neurotrophin response such as nerve growth factors (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and NT4. All these proteins bind and activate specific Trk receptors, belonging to receptor tyrosine kinase families. Neurotrophin-Trk endosomes are internalized and able to activate different intracellular pathways like phosphatidylinositol 3-kinase mediated pathways (PI-3 kinase), Ras/MAPK mediated pathways, and phospholipase Cγ (PLCγ) mediated pathways (Yamashita & Kuruvilla, 2016). Trk receptors that are bound to neurotrophic factors will initiate the formation of clathrin-mediated endosomes. The activation of Trk receptor initiates a cascade of molecular events and among them is a binding of phospholipase Cγ (PLCγ) which facilitates a recruitment of components of endocytic machinery (Bodmer, Ascan, & Kuruvilla, 2011). The internalized Trk receptor remains bounded to neurotrophic molecules, also tyrosine phosphorylated and the interior of the vesicle contains number of signalling molecules such as PLCγ, PI3 kinase and proteins of MAP kinase pathway. The vesicle is then transported to the soma through the micotubule-dynein retrograde

transport (Du et al., 2003; Yamashita & Kuruvilla, 2016). This enables neurotrophins to act on long-range signalling and also affect transcriptional events (Cosker & Segal, 2014).

Endocytosis at the synapses have a crucial role. When the neurotransmitters are released in the synaptic cleft by exocytosis, it is followed by a fast retrieval of constituent membranes and proteins. Two mechanisms have been identified in vesicle retrieval: clathrin-mediated endocytosis and bulk retrieval (Royle & Lagnado, 2003). Several studies have shown that the time scale of these two mechanisms are much different (Figure 1.3) (Xue et al., 2012). The bulk endocytosis or a clathrin-independent endocytosis is termed as a fast mode and the process takes about a second. The clathrin-mediated endocytosis, on the other hand, is described as a slow mode taking about tens of seconds (Gross & Gersdorff, 2016).

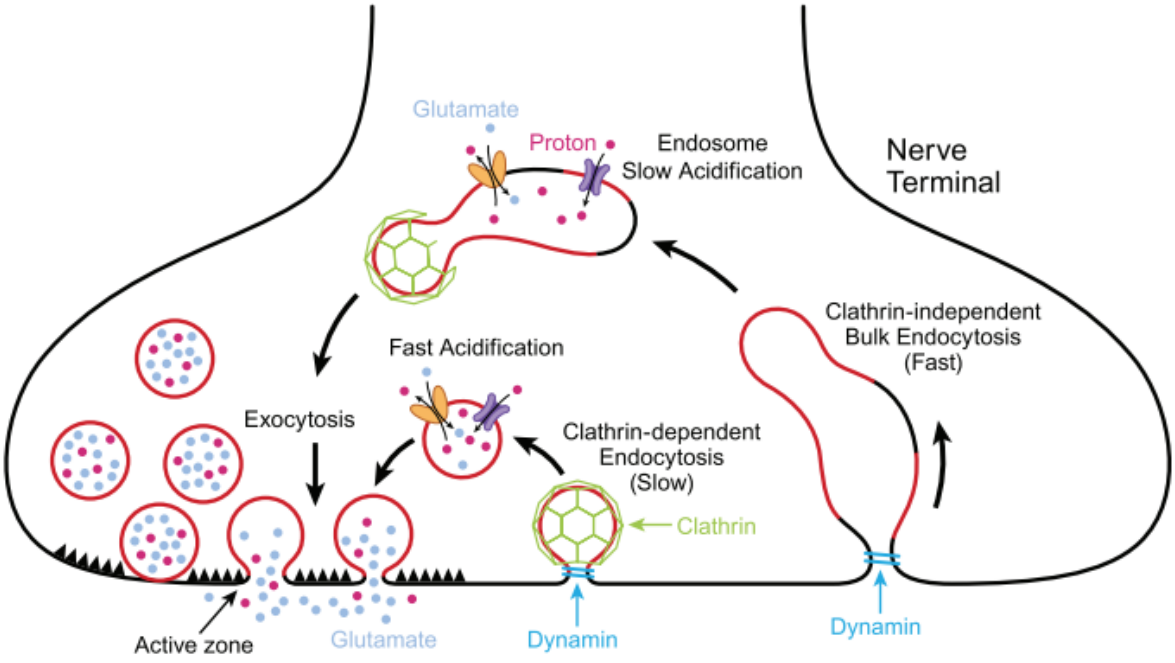


Figure 1.3. Figure illustrating endocytosis and exocytosis at nerve terminals. In exocytosis, synaptic vesicles (red circles) fuse with the cell membrane at active zone (AZ) and release neurotransmitters (red and grey dots) to the synaptic cleft. SVs that are fused are then recycled to create a new storage of readily releasable SVs at AZ. Recycling involves two processes: fast endocytosis and slow endocytosis and both pathways require protein transporters and clathrin-mediated endocytosis to create new synaptic vesicles (Gross & Gersdorff, 2016).

Different authors have shown that when neurons are moderately stimulated there are both slow and fast mode of endocytosis present in the nerve terminals, but the contribution of fast endocytosis increases progressively by stronger stimulus (Renden & Gersdorff, 2020). But when stronger and more prolonged stimulus is present, the fast mode of endocytosis, a clathrin-

independent pathway, dominates (Okamoto et al., 2016). Additionally, it has been shown that mitochondrial Ca^{2+} uptake is related to the presynaptic endocytosis. A study published in 2016 (Roslin et al.) demonstrated that the synaptic vesicle endocytosis increases when the mitochondrial calcium uniporter (MCU) channel is blocked and slows down when MCU was overexpressed. MCU is the primary calcium channel in mitochondria that is responsible for accumulating calcium ions in mitochondria at presynaptic terminals. The reason of this relation is still not clear but can be explained in the following way: ablation of calcium storage increases the acidification in the cytosol and synaptic vesicles which in turn enhances the endocytosis of synaptic vesicles. Authors of the given report also pointed out that it also shows a direct interaction between mitochondrial calcium uptake and SV endocytosis. This hypothesis could be supported by the close proximity of mitochondria and transmitter vesicles in presynaptic terminals where chemical changes can have a rapid effect on vesicles kinetics (Marland et al., 2016).

1.3. Intersectins family and their role in clathrin-mediated endocytosis

As it was mentioned earlier, ITSNs have been shown to mediate endocytosis. Besides that, ITSNs have a regulatory role also in signalling pathways such as MAPK and interact with family of Ras GTPases. Additionally, they are one of the regulators of actin cytoskeleton rearrangement and act as a bridge between receptor tyrosine kinase signal transduction pathways and clathrin-mediated endocytic internalization pathways (Herrero-Garcia & Bryan, 2018). Nevertheless, the best known function that most of the studies have focused on is the ITSNs interactions with endocytic complex where they function as adaptor and scaffold proteins for other relevant biomolecules that bind to the site (Wong et al., 2012).

1.3.1. Genetics and structure of intersectins

ITSNs are highly conserved proteins in all metazoans with orthologs from nematodes to mammals. In vertebrates, there are two genes that encode those proteins - *ITSN1* and *ITSN2* genes. *ITSN1* gene is located on the Chromosome 21 and *ITSN2* located on the Chromosome 2.

There are two main isoforms of both ITSN1 and ITSN2 – a short (ITSN1/2-S) and a long (ITSN1/2-L), which are generated by alternative splicing (Herrero-Garcia & Bryan, 2018). The two main isoforms encoded from *ITSN1* gene are respectively 1220 (ITSN1-S) and 1721 (ITSN1-L) amino acids long (Guipponi et al. 1998). While the main isoforms from gene *ITSN2* are 1249 (ITSN2-S) and 1696 (ITSN2-L) amino acids long (Pucharcos et al. 2000).

All ITSN isoforms contain two Eps15 homology (EH) domains that binds the epidermal growth factor receptor (Confalonieri et al. 2002). EH domain interacts with target proteins such as Epsin that contain a three amino acid motif Asp-Pro-Phe (NPF) motif. There are also 5 repeats of Src homology 3 domains (SH3) that have a variety of functions in endocytosis. For example, it is responsible for binding to dynamin, synaptojanin, actin and it is involved in cell signalling in general (Herrero-Garcia & Bryan, 2018). Between these two regions there is a domain called coiled-coil (CC) that promotes homo-and heterodimerization of proteins (including ITSNs) through the hydrophobic forces between α-helices (Figure 1.4). Several sequential repeats of α-helices coil together and form a structurally very stable arrangements that resemble a bundle of rope (Liu et al., 2006).

Long isoforms of ITSNs (ITSN-L) have three additional domains in their structure: Dbl-homology (DH) domain, pleckstrin-homology (PH) domain and C2 (Ca2+ binding) domain (Figure 1.4). DH and PH domains interact with Rho-family GTPases. Although it is characterized as a lipid binding region, in the case of ITSN1/2-L these domains have been shown to specifically bind and activate Cdc42 which is also a Rho-familiy GTPase. Cdc42 is known as an important regulator of cell cycle and cell division. Finally, the C2 domain is also partially implicated in GTPase regulation and membrane trafficking. (Chhatriwala et al. 2007; Herrero-Garcia & Bryan, 2018).

Previous studies have shown that the majority of ITSN1/2 isoforms (ITSN1-S, ITSN2-S, ITSN2-L) are ubiquitously expressed in many tissues and localized throughout the cytoplasm or in a perinuclear region (Herrero-garcia & Bryan 2018). However, ITSN1-L is shown to be expressed predominantly in brain (Hussain et al., 1999).

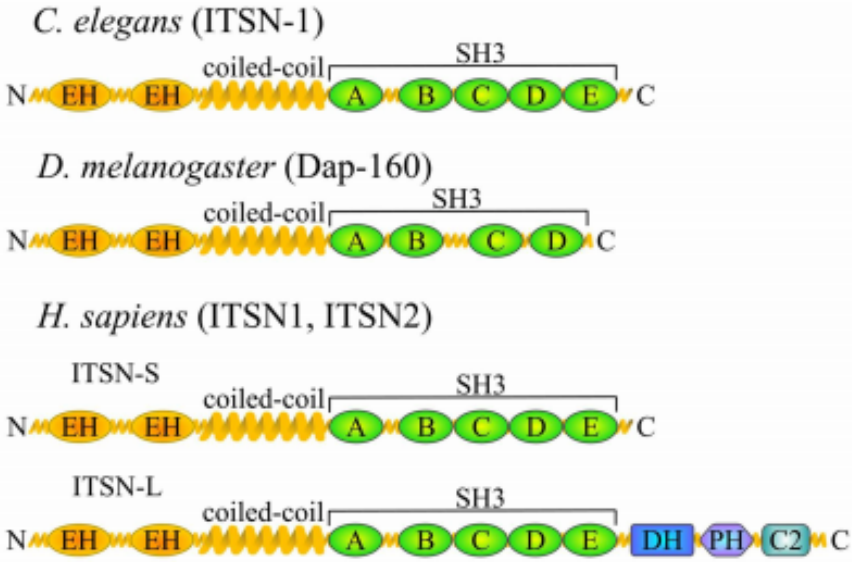


Figure 1.4. ITSN orthologs, isoforms and domain composition. ITSN proteins in different species (*C. elegans, D. melanogaster* and *H. sapiens*) have similar domain composition with conservative protein sequences. All ITSNs have EH (Eps15 homolgy), SH3 (Src homology 3) and coiled-coil domains. ITSN-L in *H.sapiens* additionally have C2 (Ca^{2+} binding), PH (pleckstrin homology) and DH (Dbl homology) domains (Gubar et al., 2013).

1.3.2. Function of Intersectins in neuronal clathrin-mediated endocytosis

As it was mentioned earlier, the best studied functions of ITSNs are their role in clathrin-mediated endocytosis. ITSNs constitute a part of the initiation complex and help to recruit essential proteins to the machinery of endocytosis, namely, dynamin, Epsin2, Eps15, Eps15L, clathrin, synaptojanin2b, Cdc42, amphiphysin, SNAP29, HIP1, Dab2, AP2 and REPs. Intersectins can also participate in GTPase activation through the DH-PH domains (Wong et al., 2012). Genetic studies have revealed that loss-of-function mutations in *C. elegans ITSN* orthologs can cause a reduced density of vesicles in synaptic terminals. That in turn suggest that ITSNs play a role in recycling of synaptic vesicles (SVs). In mammals, silencing of ITSNs lead to the defects in endocytosis although not lethal. Interestingly, in *Drosophila*, knock-out mutations in ITSN homologue *dap160* lead to the death of an animal (Herrero-garcia & Bryan 2018).

While ITSN1-L isoform expression is neuron specific, ITSN1-S is also found to be expressed in the brain but restricted to glial cells (Yu et al., 2008). Important to note that ITSN1-L knockdown studies have been observed no differences in synaptic vesicle (SV) recycling in

pre-synaptic hippocampal neurons compared to wild types. Additionally, double labelling immunofluorescence with antibodies against synaptophysin, an established marker of synaptic boutons, have shown little or no localization of intersectin1-L in axonal terminals. This data suggests that intersectins are not present in pre-synaptic axonal boutons in mammalian hippocampal neurons (Ritter et al., 2009). Contrary to these statements, in 2017, researchers from Yale University demonstrated exactly opposite data (Gerth et al., 2017). Gerth and his colleagues screened the presynaptic endocytic machinery in hippocampal neurons with mass spectrometry and immunoblotting and observed the presence of ITSN1-L in the nerve terminals. The main difference with previous study lays down in the usage of antibodies against presynaptic protein synapsin I that is known to be an abundant presynaptic protein. Immunoblotting revealed ITSN1-L as a synapsin binding partner that co-localizes at the synapses of hippocampal neurons. Further investigation showed that synapsin I binds to the SH3-A domain of intersectin 1-L. Mouse models with intersectin 1 and intersectin 2 double knockouts demonstrated that there were no significant differences in the total synaptic vesicle (SV) densities of docked SVs which is also consistent with the previous study. Additionally, double knockouts demonstrated the shift of the synapsin I localization from the centre of active zone at presynaptic terminals. This data suggested that intersectins at presynatpic terminals regulate the nanoscale distribution of synapsin I at the active zone and subsequently control the SV replenishment from the reserve pool at mammalian synapses (Gerth et al., 2017). The role of ITSN1-L or ITSNs in general at the presynaptic terminals still remains an open and ongoing question, but latest studies tend to suggest that ITSNs are indeed present at the site and therefore need to have a function there.

Currently, localization of ITSN1-L at the postsynaptic dendritic spines and somas is an established concept. Previous studies have found that intersectins co-localize with AP2 protein in dendrites and clathrins at the clathrin-coated pits in a somatodendritic regions. Analysis of the rate of clathrin-mediated endocytosis with transferrin has additionally demonstrated that hippocampal neurons with ITSN1-L knockdowns have a significant reduction in transferrin uptake and a constitutive endocytosis in dendrites and somas. Obtained results strongly indicate that ITSN1-L functions in a clathrin-mediated endocytosis between dendritic spines and somas. Lastly, loss-of-function studies have demonstrated that ITSN1-L knockdown neurons have an increased number of long, thin and morphologically immature looking spines compared to wild type ones. These data suggests the influence of intersectins on the maturation of dendritic spines in brain tissue (Ritter et al., 2009).

Besides interacting with AP-2 and clathrin, another important binding partner is a protein Numb. This protein is shown to be localized in dendritic spines where it regulates spine development in hippocampal neurons as well as promotes neuronal polarization. Numb binding with ITSN1-L stimulates GTPase activity by interaction with Cdc42 which is consistent with the fact of an essential role of Cdc42 as part of a family of small GTPases that are essential in axonogenesis and neurite outgrowth during brain development (Hall & Lalli 2010; Herrero-Garcia & Bryan 2018).

1.3.3. Role of intersectins in neurodegenerative diseases

Due to their role in initiating clathrin-mediated endocytosis, ITSN1-L and ITSN-S are associated with multiple neurodegenerative diseases, primarily the Down Syndrome (DS) phenotype and Alzheimer's Disease (AD). *ITSN1* is located on the chromosome 21q22.2 which is the region closely linked to DS. In DS, *ITSN1* has three copies and resulting in overexpression of ITSN1-S. Additionally, *ITSN1* gene localizes in close proximity to amyloid β gene, which is also overexpressed in DS patients resulting in the development of AD-like neuropathology in mid-life which manifests in cognitive decline (Herrero-Garcia & Bryan, 2018; Rembach et al. 2019).

The research, on whether *ITSN1* is a causative factor in these pathologies, is still on going and it is still not clear if *ITSN1* actually causes neuronal disorders. Different reports and data can be contradictory (Scappini et al. 2007; Nixon, 2005; Wilmot et al. 2008; Rembach et al. 2019) or focus on different aspects on this issue. Transcriptional study on *ITSN1* expression and cognitive decline have identified significant upregulation of this gene but interestingly it has been seen specifically for short isoform, ITSN1-S. On the same study, this transcript was confirmed to be upregulated in AD models (Wilmot et al., 2008).

In the study published in 2019, protein expression levels of ITSN1-S and ITSN1-L isoforms in human post-mortem brain tissues were analysed. No differences between the levels of ITSN1 proteins (both L and S isoforms) in frontal and temporal cortices of AD patients compared to the healthy control ones was reported in this study (Malakooti et al., 2019).

1.4. microRNAs: biogenesis, mechanism of action and dysregulation in neurodegenerative diseases

MicroRNAs (miRNAs/miRs) are a large group of non-coding RNAs that regulate the expression of many protein-coding genes by binding with messenger RNAs (mRNAs). miRNAs were discovered in 1993 (Lee et al. 1993) although their function as regulators of gene expression and involvement in RNA interference (RNAi) was first observed in late 1990s (Fire et al. 1998) which later brought a major shift in our understanding of gene regulation and Nobel prize in medicine and physiology to the authors. More than a decade of microRNA research has demonstrated that these small non-coding RNAs are extensively used by living organisms to regulate gene expression (O'Brien, Hayder, Zayed, & Peng, 2018).

Mature miRNAs are short non-coding molecules with an average length of 22 nucleotides (Weiss & Ito, 2018). miRNA maturation usually includes several processing steps. Firstly, they are transcribed from miRNA-coding genes as relatively long (several hundred nucleotides) primary miRNAs (pri-miRNA) by RNA polymerase II or III (O'Brien et al., 2018). In some cases, pri-miRNAs can be transcribed as one long transcript containing several mature miRNA sequences, called miRNA cluster (Kabekkodu et al., 2018). Next, pri-miRNAs are processed by microprocessor complex containing RNA binding protein DGCR8, which recognizes the RNA, and ribonuclease III enzyme Drosha that cleaves pri-miRNA duplex at the base of its hairpin structure. The resulting 70 to 100 nucleotides molecule, pre-miRNA, is exported to the cytoplasm by exportin5/RanGTP complex with subsequent cleavage by RNAse III endonuclease Dicer. Endonuclease removes the loop structure from the molecule resulting in a mature microRNA molecule. The final cleavage creates two single strands of miRNA – with the first one generated from the 5' end of pre-miRNA (5p strand) while the second comes from the 3' end (3p strand). Next, mature miRNA has to bind with Argonaute protein to guide the regulation of mRNAs. In principal, both strands can be loaded onto the Argonaute (AGO) protein but usually the strand with lower 5' stability is loaded into AGO and guides Argonaute to the target messenger RNA that contains complementary sequence to the miRNA seed region while the second strand is degraded (O'Brien et al., 2018).

The complex of miRNA guiding strand and AGO is called miRNA-induced silencing complex (miRISC) and the complex can either silence gene expression by inhibiting protein translation or by promoting mRNA decay (Behm-Aansamant et al. 2006). The degree of complementary of guiding strand to target mRNA determines whether the target strand is degraded by AGO2-dependant manner or translationally inhibited by miRISC complex. In

mammals, most of miRNA and target interactions are not fully complementary which means they are only translationally inhibited. Most of the studies have also shown that miRNAs bind to the specific sites at 3' untranslated region (UTR) of target mRNA. The binding to the 3'UTR induces translation downregulation of the given mRNA. Other binding sites for miRNA, but less important, can be located at 5'UTR, coding region and also promoter region in DNA. Binding to 5'UTR or coding region also exhibits silencing effects on the mRNA. On the other hand, interaction with promoter region induces transcription (O'Brien et al., 2018).

miRNA systematic studies have demonstrated that many miRNAs are significantly up- or downregulated in many neurodegenerative diseases such as amyothrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease and multiple sclerosis. The dysregulation of miRNAs in turn disrupts the regulation of receptors and enzymes. Previous studies have shown the interactions between miR-21-5p and amyloid-β, α-synuclein and T-cell activation. Also, regulations of NF-κB by miR-146a-5p and glutamate receptor subunit GluR2 by miR-223-3p have been demonstrated (Juźwik et al., 2019).

So far, little is known about the intersectins regulation and the effect of miRs on the expression of intersectins. The aim of our study was to investigate the regulation of ITSNs by microRNAs in HEK293T and human neuronal cell culture.

Aims of the study

The objectives of the study were following:

- Create plasmid luciferase reporter constructs bearing 3'UTRs of *ITSN* genes and microRNAs. Transfect HEK293T cells with constructed plasmids and analyse the expression levels of luciferase, reflecting on which microRNAs have the most significant down or upregulating effect on 3'UTRs.
- Construct lentivirus particles containing microRNAs. Transduce human neuronal cell cultures with virus particles and analyse intersectins transcripts (mRNA) levels by RT-qPCR.

2. Materials and Methods

2.1. Culturing of cell lines

2.1.1. HEK 293T

Human embryonic kidney (HEK) 293T cell line was cultured in a DMEM growth medium (Dulbecco's modified eagle's medium), 10% of fetal bovine serum (FBS) and 0.1 mg/ml of normocine antibiotic to prevent bacterial contamination and mycoplasma growth. Before passaging, the cells were washed with 4 mL of 1x phosphate-buffered saline (PBS) solution. Then, the cells were treated with 1 ml of trypsin for 2 minutes to detach them from the surface of a culturing flask and suspended in DMEM media 2x of the volume of trypsin. Cell cultures were incubated either in 50 ml culturing flask or in 10 cm Petri dish (Cellstar) in a cell culture incubator at 37°C and 5% CO_2.

2.1.2. Human neuronal stem cells

In addition to HEK293T cell line, we used human neuronal stem cells (NSCs) derived from cell line HEL47.2. This human iPSC cell line was derived from the dermal fibroblasts of the 83-year old male (Trokovic et al. 2015). NSCs were first cultured on a 3.5 cm Petri dishes and then passaged on the 12-well plate. First, the wells were coated with basement membrane matrix. Corning Matrigel^{TM} GFR membrane matrix aliquots (100 µl) were dissolved in a cold DMEM/F12 medium in a ratio of 1:200. 1.5 ml of the solution was added to the 3.5 cm Petri dishes and 1 ml to each well in 12-well plates. NSCs were cultured in a neuronal differentiation medium (NDM) [DMEM/F12 and Neurobasal medium (Gibco) in a ratio 1:1, B-27 (1:100) (Gibco), N-2 (1:200) (Gibco), 2mM of glutaMAX (Thermo Fisher Scientific) and 10 µg/ml of Penicillin-Steptomycin (PenStrep, Thermo Fisher Scientific) antibiotics]. After thawing of NSCs vials from liquid nitrogen, they were gently transferred into 4 ml of NDM medium and centrifuged at 500 rpm for 5 minutes. Then, discarding supernatant, resuspending the pellet in 1 ml of NDM and adding 10 µM of ROCK inhibitor (Rho kinase inhibitor). Finally, cell suspension was gently pipetted to the prepared Petri dishes. Next day, the medium was changed to remove dead cells and ROCK inhibitor.

Before passaging to the 12-well plates, NSCs were incubated for 3-5 days to form networks. The time period between the passaging of NSCs from 3.5 cm dishes to 12-well plate was estimated based on the cell recovery which was assessed under the microscopy DM IL (Leica, Germany). NSCs were let to mature and grow processes which assured that they were indeed in good condition which approximately took 3-5 days. Dishes with the NSCs were first washed with 1 ml PBS, then treated with 1 ml of accutase and incubated for 8 minutes at 37°C. Then accutase-treated cells were carefully rinsed with 3x1 ml of NDM per dish to detach cells from the matrix, transferred to the 15 ml Falcon tubes and centrifuged at 300 x g for 3 minutes. The pellet was resuspended in 1-3 ml of NDM and slowly pipetted up and down. Then, cell suspension was pipetted through the cell strainer, to get rid of cell clumps. After that, 10 µl of cell suspension was mixed with 10 µl of trypan blue (Thermo Fisher Scientific) and taken to the haemocytometer to calculate an approximate number of cells. For culturing cells, cell density should be approximately 100000 cells/cm^2. Meaning, in one well, in 12-well plate with well radius of 1.105 cm, the approximate number of cells would be 400000 million of cells per well. The medium was changed every 2-3 days.

2.2. Design of primers and plasmids

2.2.1. Design of miRNA oligonucleotides and 3'UTRs cloning primers

Primers for both miRNAs and 3'UTR regions of intersectins were ordered from Metabion International AG (Germany) in lyophilized or water-diluted form (100 µM concentration). Primers for 3'-untranslated regions (3'UTRs) were designed to clone 3'UTRs of ITSN2, ITSN1-L and ITSN1-S. Because ITSN1-L has exceptionally long 3'UTR region (11556 bp) (Kropyvko, 2010), it was divided into three segments – ITSN1-LA, ITSN1-L-B and ITSN1-L-C. Therefore, primers were separately designed for each part of ITSN1-L 3'UTR region to clone them separately to the expression vectors. To obtain 3'UTRs from the human male genomic DNA (Promega, USA), PCR reaction with H_2O, 1xHighFidelity (HF) buffer, 1 µM dNTP, 10 µM forward and reverse primers, Phusion DNA pol (Thermo Fisher Scientific) and template DNA was performed with the following program parameters: denaturation at 98°C for 10 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 30 seconds, which was repeated in 25 cycles (SimpliAmp Thermal Cycler, Applied Biosystems). After that, samples

were cooled down to 4°C and stored at -20°C. The sequences of all primers are shown in Table 1.1.

Primers of 3'UTR regions	**Sequences (5'-3')**
3'UTR ITSN1-S For	TAGGCGATCGCTCGAATCATATGTTGTCCATCCC
3'UTR ITSN1-S Rev	TTGCGGCCAGCGGCCTAACTTTTGTATGAAGAATTTAA
3'UTR ITSN1-L A For	TAGGCGATCGCTCGAGCAGCGGGCTCAGGGTGT
3'UTR ITSN1-L A Rev	TTGCGGCCAGCGGCCCCTCTTATGGTGGCTCAGACT
3'UTR ITSN1-L B For	TAGGCGATCGCTCGACCTTGTATAAGCAGAAAAAGACTTC
3'UTR ITSN1-L B Rev	TTGCGGCCAGCGGCCGAGCTCAATGGTAAAGGCTCAC
3'UTR ITSN1-L C For	TAGGCGATCGCTCGAGTTGCTCCACAGTCCTGTC
3'UTR ITSN1-L C Rev	TTGCGGCCAGCGGCCTTGGCTTTTCTCCTTTTATTCATG
3'UTR ITSN2 For	TAGGCGATCGCTCGAGGGTTCTAAAGGACAGCAC
3'UTR ITSN2 Rev	TTGCGGCCAGCGGCCTTCCATCCATAAACATATGTCTTT

Table 1.1. Primers to amplify 3'UTR's of ITSN genes. Amplified regions were then ligated to the psiCHECK™2 Vector (Promega, USA).

MicroRNA single stranded oligos were designed for human microRNAs: miR-30a-5p, miR-19, miR-124-3p, miR-128, miR-181a-5p and for miR-218 and ordered from Metabion International AG (Germany) in lyophilized of 100 μM water-diluted form. Sequences of microRNA oligos are shown in the Table 2.

Primer for miRNA cloning	Sequences (5'-3')
miR-30a-5p top	TGCTGTGTAAACATCCTCGACTGGAAGGTTTTGGCCACTGACTGA CCTTCCAGTGGATGTTTACA
miR-30a-5p bottom	CCTGTGTAAACATCCACTGGAAGGTCAGTCAGTGGCCAAAACCT TCCAGTCGAGGATGTTTACAC
miR-124-3p top	TGCTGTAAGGCACGCGGTGAATGCCAAGTTTTGGCCACTGACTG ACTTGGCATTCGCGTGCCTTA
miR-124-3p bottom	CCTGTAAGGCACGCGAATGCCAAGTCAGTCAGTGGCCAAAACTT GGCATTCACCGCGTGCCTTAC
miR-181a-5p top	TGCTGAACATTCAACGCTGTCGGTGAGTGTTTTGGCCACTGACTG ACACTCACCGACGTTGAATGTT
miR-181a-5p bottom	CCTGAACATTCAACGTCGGTGAGTGTCAGTCAGTGGCCAAAACA CTCACCGACAGCGTTGAATGTTC

Table 1.2. Oligos for microRNA cloning. Single-stranded oligos were used to generate double-stranded ones that were then ligated to the pcDNA6.2-GW/EmGFP vector backbone.

2.2.2. Construction of miRNA expressing plasmids

For microRNA cloning, top and bottom single-stranded oligos for each microRNA were annealed to generate double-stranded insertion containing microRNA sequence (Table 1.2). For annealing single-stranded oligos, 50 µM of both top and bottom strand, 1x annealing buffer (10x) (10 mM Tris, 50 mM NaCl, 1 mM EDTA) were added to the reaction, which was incubated on a thermomixer 5436 (Eppendorf, Germany) for 4 minutes at 95°C. After incubation, the reaction was cooled down to room temperature (RT) and stored in a freezer at -20°C. Double-stranded miRNA oligos were ligated to the commercially available expression vector pcDNA^{TM}6.2-GW/EmGFP (Figure 1.3) (Invitrogen, Thermo Scientific, USA). The given vector simultaneously expresses Emerald Green Fluorescent Protein (EmGFP) which allows to track cells under fluorescence microscopy (EVOS^{TM} FLoid Thermo Fisher Scientific, USA) that express given expression vector. A ligation reaction was preformed with the following reagents: 1x ligation buffer (10x) (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT), 1 µl of pcDNA^{TM} 6.2GW/EmGFP expression vector, 10 µM double-stranded oligos

and 1 U/µl of T4 DNA ligase in milliQ water and incubated at RT for 30 minutes and used for *E. coli* transformation.

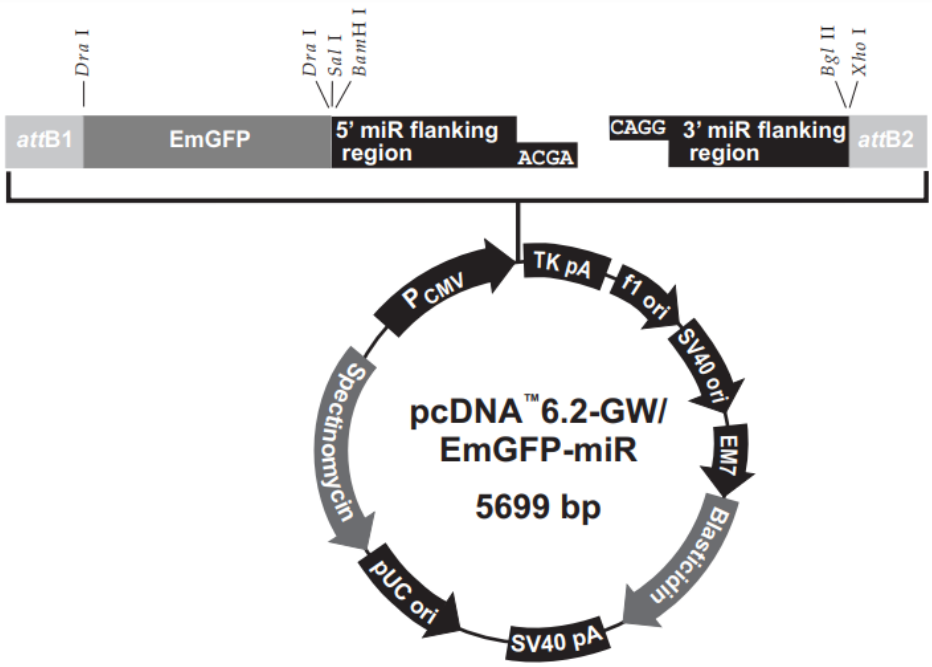


Figure 1.3. pcDNA™6.2-GW/EmGFP-miR vector for microRNA expression. Selected microRNA sequences were ligated in the region depicted above using sticky ends of ACGA and CAGG. EmGFP gene (Emerald Green Fluorescent protein) expression was used to confirm successful transfection of the construction in transfected cells under green light. *Spectinomycin* resistance gene was used to selectively cultivate *E.coli* cells transformed with the given vector (Invitrogen™, 2010).

2.2.3. Construction of plasmids bearing 3'UTRs of ITSN1/2

For the expression of ITSN 3'UTRs (target DNA), I used a commercial psiCHECK™-2 Vector (Promega), which is optimized to perform RNAi assays (siCHECK™ Vectors, 2016). After performing PCR with target primers, PCR products were run on 1% agarose gel [≈0.5 µg/ml ethidium bromide, 1xTAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) for 150 ml] to visualize the PCR products under UV-light and excise appropriate bands from the gel.

PCR fragments were then extracted from the gel using a commercial NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). First, NTI solution was added to the gel fragments with a 2x volume (µl) of the mass (mg) of the gel fragments. Gel fragments with NTI solution were then

incubated at 50°C for 10 minutes and transferred to the NucleoSpin extraction column tubes. Tubes were spinned in a centrifuge 5702 (Eppendorf) for 30 seconds at 11 000 g. Then washed 3x with 700 µl of NT3 solution and centrifuged again after each wash at the same parameters. Then, columns with PCR products were dried by centrifugation without adding any solution and heated for 2 minutes at 70°C on a Thermomixer 5436 (Eppendorf). To elute DNA from columns, 30 µl of pre-warmed elution buffer at 70°C were added, incubated for 5 minutes at 70°C and centrifuged down at 11 000 g for 1 minute. The concentrations were measured on a spectrophotometer NanoDrop ND-1000 (Thermo Scientific, USA).

After gel extraction, PCR fragments were ligated to the psiCHECK™-2 expression vector (Figure 1.4) using a 2x NEBuilder High Fidelity (HiFi) DNA Assembly master mix (New England BioLabs Inc., USA). Then, 15-30 ng/µl of PCR fragments and 50 ng of linearized vector (treated by XhoI and NotI restrictases) were added to the master mix and incubated at 50°C for 15 minutes. After incubation and cooling on ice, ligation mix was used for *E. coli* transformation immediately or stored at -20°C.

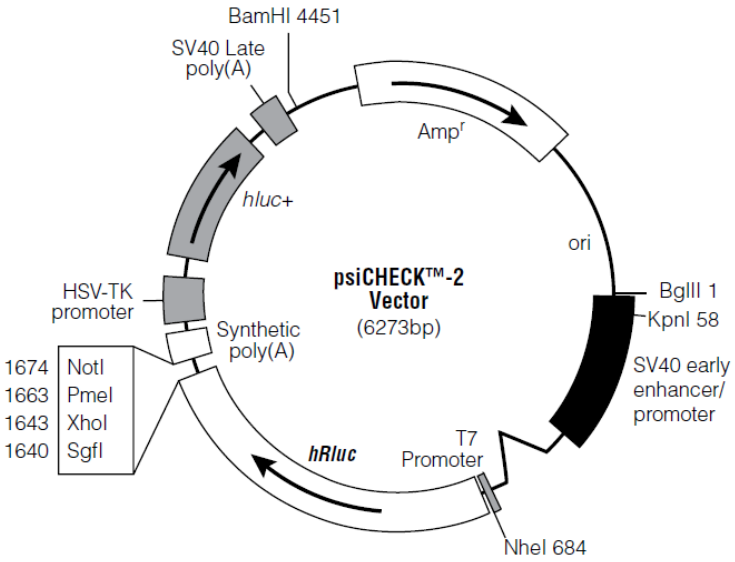


Figure 1.4. PsiCHECK™-2 expression vector for cloning 3'UTRs of ITSN mRNAs. 3'UTRs were ligated to the vector linearized with NotI (1674) and XhoI (1643) restriction enzymes to be transcribed together with *hRluc* (*Renilla* luciferase) gene regulated by SV40 promoter. Selection of positive *E. coli* clones was performed using ampicillin resistance gene (*Amp*^{r}) (siCHECK™ Vectors, 2016).

2.2.4. Construction of lentivirus transfer plasmids for miRNA expression

Lentivirus-based miRNA expressing vectors were made based on the pCDH-hSYN backbone with a human synapsin (hSyn) gene promoter characterized by highly neuron-specific long-term transgene expression properties. hSyn promoter can restrict transgene expression only to neurons (Kügler et al. 2003). EmGFP-miRNA region, obtained from the pcDNA^{TM}6.2-GW/EmGFP-miR vector (Fig. 1.3), was ligated to the backbone to express selected miRs and Emerald Green Fluorescent Protein (EmGFP) to confirm microRNA expression under fluorescence microscopy EVOS^{TM} FLoid (Thermo Fisher Scientific, USA). pCDH-hSyn-MCS-T2A-EGFP plasmid was digested with BamHI and SalI restriction enzymes. Digestion reaction contained 1xFD buffer (Fast Digest, Thermo Fisher Scientific), 0.5 µl (0.5 U) of restriction enzymes and 3 µl of target plasmid that were diluted in water to obtain 15 µl of final volume of the reaction. The reaction mixture was incubated at 37°C for 30 minutes and then run on 1% agarose gel electrophoresis (1xTAE buffer, ≈0.5 µg/ml ethidium bromide). The correct band from the gel was then excised and purified using the same manual from the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) as described above. Next, EmGFP-miR region from the pcDNA^{TM}6.2-GW/EmGFP-miR vector was amplified in PCR reaction with primers listed in the Table 1.5 to ligate it to the backbone. Obtained PCR fragments were then ligated to the purified and linearized backbone using 2x NEBuilder High Fidelity (HiFi) DNA Assembly master mix (New England BioLabs Inc., USA) as described above and transformed to the NEB 5-α competent *E.coli* cells (New England BioLabs Inc., USA). Positive clones were selected and used for plasmid DNA isolation with subsequent digestion analysis and sequencing to confirm successful construction of the new miRNA-bearing plasmids. In total, constructions bearing hsa-miR-181a-5p, hsa-miR-30a-5p, hsa-miR-124-3p and negative control microRNA (does not target any known gene in the human genome) were generated.

Primers for EmGFP-miR region	**Sequence (5'-3')**
EmGFP For	GTATAAACTTAAGCTTCACCATGGTGAGCAAGGGC
miR Rev	CAGCGGGTTTAAACGGGCCCCTCGAGTGCGGCCAGATCT

Table 1.5. Primers to amplify EmGFP-miR region from pcDNA^{TM}6.2-GW/EmGFP-miR vector. Both primers have overlaps of 15 nucleotides (nt) with the pCDH-hSyn backbone. Successful recombination of the EmGFP/miRNA sequence with pCDH-hSyn vector caused deletion of BamHI restriction site with keeping SalI restriction site in the final construct.

2.3. Transformation

After obtaining correct expression vectors, they were used for transformation of *Escherichia coli* (*E.coli*). For the transformation, we used 50 µl aliquots of NEB 5-α competent *E.coli* cells (New England BioLabs Inc., USA). 1-2 µl of plasmids were added to the aliquot and left on ice for 30 minutes. Then, placed to the heat block for 45 seconds (at 42°C) and put back on an ice for 5 minutes to recover. After that, we added 450 µl of SOC outgrowth medium (New England BioLabs Inc., USA) and incubated for 1 hour at 37°C on a shaker Thermomixer 5436 (Eppendorf AG, Germany).

After incubation, the suspension was plated on 10 cm Petri dishes with LB medium (lysogeny broth). miRNA-containing vectors were plated on LB dish with 0.05 mg/ml spectinomycin antibiotic while vectors with fragments of 3'UTRs were plated on dishes containing 0.1 mg/ml ampicillin in LB agar. All dishes were incubated on 37°C for overnight. Next day, a single colony was taken out from the dish and inoculated to 2 ml liquid LB medium (0.1 mg/ml ampicillin or 0.05 mg/ml spectinomycin) and incubated overnight on 37°C on a shaking platform Polymax 1040 (Heidolph, Germany).

2.4. Isolation of plasmid DNA, digestion analysis and sequencing

Plasmid DNA was isolated using a NucleoSpin Plasmid kit (Machery-Nagel, Germany). After overnight growth, the cell cultures were transferred to 2 ml Eppendorf tubes and centrifuged at 11 000 g for 1 minute. Supernatant was removed and 250 µl of A1 (+RNAse) solution was added to the cell pellet. Then, it was briefly vortexed to mix it and 250 µl of A2 buffer was added to the mixture. Samples were then incubated at RT for 5 minutes. After that, 300 µl of A3 solution was added and the mixture was shaken in hand untill it turned colourless. Then, samples were centrifuged in a Heraeus Pico 17 centrifuge (Thermo Scientific, USA) for 5 minutes at 11000 g. After centrifugation, plasmid DNA remained in a supernatant was

transferred to the NucleoSpin column tubes. Next, the samples were centrifuged again at same conditions for 1 minute. The flow-through in a collecting tube was discarded and 500 µl of AW (washing) buffer was added to the columns. It was again centrifuged at same conditions as before and flow-through was again discarded. Now, 600 µl of A4 (with ethanol) buffer was pipetted to the columns and samples were centrifuged again. After that, the supernatant was discarded again and the samples were centrifuged once again (2 minutes, 11 000 g) to dry. Finally, the columns were carefully transferred to 1.5 ml Eppendorf tubes and 50 µl of AE (elution) buffer was pipetted to the column filters. Then, samples were incubated for 2 minutes on 70°C and centrifuged down at 11 000 g for 1 minute to elute plasmid DNA. The concentration of DNA was measured on a NanoDrop ND-1000 (Thermo Scientific, USA).

2.5. Transfection of HEK293T

When HEK293T cells became confluent on the 96-well plate they were ready to be transfected. The transfection scheme was made so that the final concentration of the 3'UTR and the microRNA-containing plasmids were both 500 ng/µL per well. Before adding vectors with target 3'UTRs and miRNAs to the plate they were all diluted to 100 ng/µL. The final volume of transfection mix in luciferase assays was 100 µl per well therefore there was 200 ng of DNA (1:1 of target DNA and miR) per well. Transfection mix included 0.4 µL of polyethyleimine (PEI), 25 µL of OptiMEM (optimal minimal essential medium) and 72.6 µL of DMEM (10% FBS) per well. After adding the transfection mix, cells were incubated for 48h at 37°C.

2.6. Production of lentiviral particles

In order to produce effective virus particles able to infect NSC we used a 3rd generation of Lentivirus production system with an addition of pAdVAntage™ vector (Promega, USA). This vector was included to the system because it expresses adenoviral virus associated I (VAI) RNA to enhance protein expression in mammalian cells. The Lentivirus production system included three plasmids. First vector pMDLg/pRRE was needed to express polymerase (HIV-1 POL) and inner shell proteins (HIV-1-GAG). Second one, pRSV/REV, expressed a Rev protein which helps to relocate newly transcribed HIV mRNA to cytoplasm. Otherwise, it would stay in nucleus and the translation would be prevented. The last one, pMD2.G, expresses VSV

glycoprotein (VSV-G) (Gandara et al., 2018). Such complex system is necessary to create biologically safe replication-deficient lentiviral vectors, which can transduce, but unable to propagate, in target cells (Milone & Doherty, 2018).

First, we seeded HEK293T cell lines to the 10 cm Petri dishes (Cellstar) in 10 ml DMEM (10% of FBS, 0.1 mg/ml normocin) and 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Cells were grown until 70-80% confluency. Vectors were added to the dishes so that the final amount of each vector is 2 µg per dish. The only difference was that the transfer vector had the amount of 4 µg per dish. DNA mixtures were mixed into the 500 µl of pre-warmed OptiMEM II medium. Then, 48 µl of PEI (diluted in 1xPBS) in a ratio of 4:1 (PEI:DNA) was added. Next, the solution was briefly vortexed and then left to incubate at RT for 5-10 minutes. After incubation, the transfection solution was pipetted to the cell cultures and dishes were swirled few times so that the media would distribute evenly. After about 72 hours of transfection, viruses were collected. Media was transferred to the 50 ml tubes and centrifuged at 200 g for 5 minutes in SL 16R centrifuge (Thermo Scientific, USA). Viruses would stay in a supernatant which was filtered through the 0.45 µm filter. Supernatant was then poured to the ultracentrifuge tubes (Beckamn UltraClear) and centrifuged in the Beckman AH-629 rotor using Evolution™ RC Superspeed Centrifuge (Sorvall, USA) for 1.5 hours at 25 000 rpm 4°C. After that, the virus particles migrated to the bottom of the tubes. The supernatant was then discarded and the pellet was resuspended in 60 µl of sterile Dulbecco's PBS. All resuspended pellets were then collected to a single 1.5 ml Eppendorf tubes and centrifuged on a benchtop centrifuge (Thermo Scientific, Pico 17) for 1 minute at maximum speed. Virus particles were stayed in a supernatant which was then collected and stored in -80°C in an aliquot tubes (11 µl per tube).

2.7. Transduction of neuronal cells

Transduction of NSCs HEL47.2 was done by first taking away the old media. Pipetting was done very slowly and carefully so that neuronal cells does not detach from the matrix. The final volume of new NDM media was 1.5 ml per well. First, we added 750 µl of new media to each well. Then another 750 µl was first mixed with a 5 µl of Lentivirus aliquots in a separate 1,5 ml Eppendorf tubes. The volume of virus stock (with the titer of $\approx 10^9$ virus particles/ml) was calculated to obtain multiplicity of infection (MOI) of ≈ 5, which theoretically should result in

transduction of 99.3% cells. After that, the mixture was also slowly pipetted to each well. Plates were then put back to the 37°C, 5% CO_2 incubator.

2.8. Dual-luciferase reporter assay

Dual-Luciferase Reporter (DLR) assay was performed using a commercial Reporter Assay System from Promega, USA. All reagents were prepared according the commercial manual of the kit. Transfected HEK293T cells were first lysed by 50 µl of passive lysis buffer (PLB) per well. Treated cells were then incubated in PLB solution for 15 minutes at room temperature on a shaking platform (PerkinElmer, United Kingdom). After that, a 10 µl from each well were transferred to the special 96-well plate, OptiPlate (PerkinElmer, USA) for luminescence measurements. For the measurements of luciferase activity, two reagents were used – luciferase assay reagent II (LARII) for firefly luciferase and Stop & Glow (S&G) reagent for the Renilla luciferase. LARII reagent was first diluted in 1:5 ratio in a sterile water. S&G reagent was first diluted in S&G buffer in a ratio of 1:50 and then diluted once more in sterile water in 1:5 ratio, so that the final ratio would be 1:250. Samples were then analysed in a multilabel analyser VICTOR^3 (PerkinElmer, USA) with a Wallac 1420 workstation software.

psiCHECK™-2 vector encodes two different bioluminescence proteins, firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*) luciferase, which have evolutionary different origins and chemically different substrate requirements. Firefly luciferase gene is constitutively transcribed and translated and produces bioluminescence which, theoretically, does not depend on microRNA and, therefore, is used to normalize the signal between the wells (Fig. 1.5). The *Renilla* luciferase gene is ligated with our desired ITSN1/2 3'UTR sequences and they are transcribed as a single mRNA. So, when the cell lines, being transfected with both vectors psiCHECK™-2 and pcDNA™6.2-GW/EmGFP-miR, bearing particular microRNA, it should bind the hRluc-3'UTR mRNA and, downregulate the production Renilla luciferase protein which is fused with the target transcript (Fig. 1.4). As a result, it would manifest as a lower bioluminescence signal (*Renilla* luciferase) when compared to bioluminescence signal produced by firefly luciferase. In the DLR assay system, firefly luciferase activity is measured first as a reference luciferase activity by adding LARII to the wells and measuring the signal. Then, by adding Stop & Glow solution, firefly reaction is quenched and *Renilla* luciferase activity simultaneously initiated and measured (Technical Manual: Dual-Luciferase® Reporter Assay System, 2015).

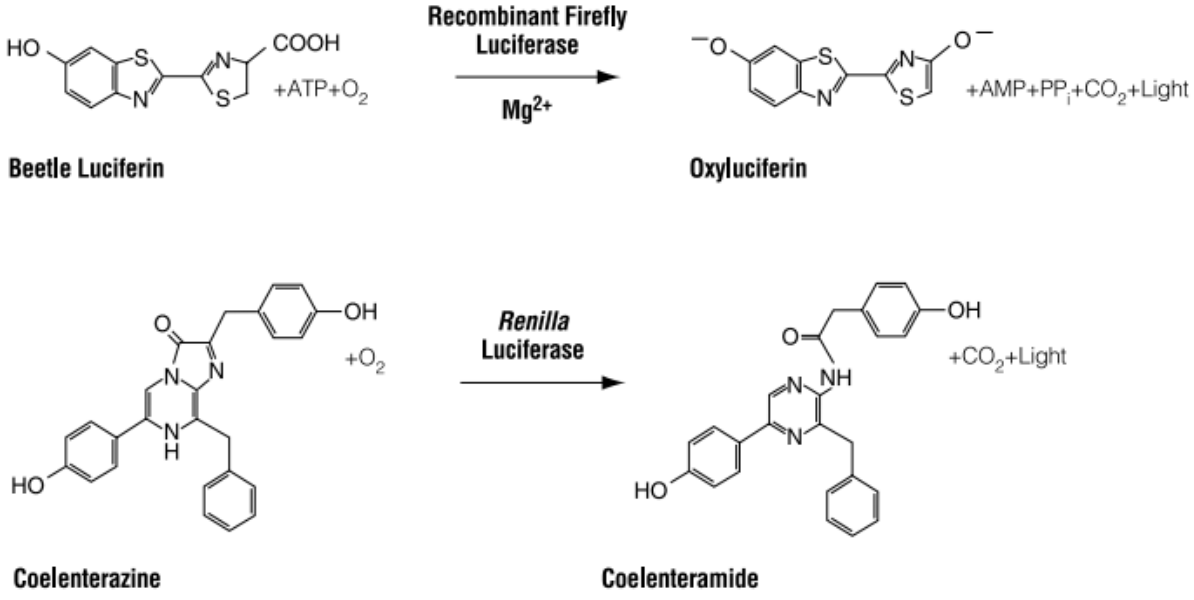


Figure 1.5. Bioluminescent reactions catalysed by firefly and *Renilla* luciferases used in DLA. Firefly luciferase reaction requires ATP, O_2 and Mg^{2+} and as a result one of the by-products is a flash of light. *Renilla* luciferase reaction requires only O_2 and also produces a flash of light as a result (Technical Manual: Dual-Luciferase® Reporter Assay System, 2015.).

2.9. RNA isolation and RT-qPCR

The cells were lysed either by adding Laemmli buffer, for ITSN protein analysis, or TRI reagent (Thermo Scientific, USA) for ITSN mRNA analysis. For the latter, I started by isolating the total RNA. For the 12-well plate, I added 350 µl of TRI reagent solution per well. Then, pipetting the solution up and down for few times to detach the cells from the surface. After that, they were transferred to 1.5 ml Eppendorf tubes. At this point, the samples could be stored at -80°C or continue with the isolation. Then, I added chloroform with a ratio of 1:5 to TRI reagent, shook it and then incubated for about 3 minutes at RT. After incubation, samples were centrifuged at 12 000 g's for 15 minutes at 4°C (Heraeus Biofuge Fresco centrifuge, Germany). After centrifugation, two layers were visible with the white solid material between them. The upper, transparent layer was transferred to new 1.5 Eppendorf tubes and isopropanol was now added (1:2 volume of TRI reagent). Next, samples were incubated at RT for 10 minutes and centrifuged again at same conditions for 10 minutes. Now, a small amount of white pellet could be visible in the bottom of the tube. Most of the supernatant was removed and 75% ethanol was added to the tubes (1:1 ratio to trizole). Samples were mixed and spinned again at 4°C, 7500 g

for 5 minutes. Supernatant was discarded and samples were spinned once again for 1 minute at same conditions. Then, the rest of the supernatant was removed and tubes were let to dry at RT with open lids for about 10-15 minutes. After complete drying, the pellet was dissolved in 50 µl of sterile RNAse-free water and stored at -80°C. Concentration of total RNA was measured on NanoDrop ND-1000 (Thermo Scientific, USA).

After measuring the concentrations of total RNAs we then synthesised complementary DNA (cDNA) in order to perform reverse transcription quantitative PCR (RT-qPCR) to assess miRNA and target expression. To synthesize cDNA for each miRNA, we used a TaqMan MicroRNA RT kit (Applied Biosystems, USA). Each RNA sample was synthesized in three technical repeats. The first one had a specific microRNA primer for the given miR, second one primer for negative control microRNA and third one as a negative control of cDNA synthesis (water). The reaction mix included 1 mM dNTP's, 50 U/µl of MultiScribe RT enzyme, 1xRT buffer, 20 U/µl of RNAse inhibitor, U6 promotor 1xRT primer, water and specific miR 1xRT primer for the synthesis of specific microRNA cDNA. Lastly, we mixed it with 10 ng of RNA sample. Samples were then mixed and centrifuged briefly to spin down all the liquid drops and then run on a thermal cycler (SimpliAmp Thermal Cycler, Applied Biosystems, USA) in a following program: 30 minutes on 16°C, 30 minutes on 42°C, 5 minutes on a 85°C and finally cooled down to 4°C. When the program was done, the samples were diluted by adding 60 µl of sterile water. Now samples could be stored in -20°C or proceed with qPCR. For target synthesis (3'UTRs) we did not need to run it in triplicates. Samples from every miR were diluted to 500 ng of total RNA, added to PCR tubes and then mixed with reagents. The reaction included 5 mM oligo dT, 5 mM dNTP mix (Thermo Fisher Scientific, USA), 500 ng of total RNA sample. Then incubated at 65°C for 5 minutes. Then put back to the ice and added 1xRT buffer and 10 U/µl of Maxima H Minus RT (Thermo Fisher Scientific, USA) which is designed to have very high processivity. Then the reaction was run in a thermocycler on a following program: 30 minutes at 50°C, 5 minutes at 85°C and then cooled down to 4°C. Then, like previously, samples could be stored in -20°C

Next, I performed RT-qPCR on a LightCycler 480 386-Multiwell Plate (Roche, Switzerland). The final volume of each sample on the plate was 10 µl and all the samples were loaded in two repeats (duplicates). For microRNA expression measuring we used a TaqMan Universal PCR Master Mix (1:2 ratio to final volume) (Roche, Switzerland), specific TaqMan small RNA primer mix and template cDNA. For targets qPCR we used LightCycler 480 SYBR Green Master mix (1:2 ratio) (Roche, Switzerland), 0.2 µM of both forward and reverse primers, PCR grade water and template cDNA (1:2.2 ratio). Samples were then transferred to

LightCycler®480 (Roche). Pre-incubation was done at 90°C for 5 minutes. Then, amplification program was following: denaturing 95°C for 10 seconds, annealing at 60°C for 10 seconds and elongation at 72°C for 10 seconds, repeated for 45 cycles. Then samples were cooled down to 40°C.

2.10. Western blotting

For the protein analysis, we performed SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and western blotting (WB). Cell cultures were lyzed by adding 100 µl of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue). Then, the cell layer was scraped off the surface and transferred to 1.5 ml Eppendorf tubes and incubated on a Heating Block MB-102 (Bioer, Germany) at 95°C for 10 minutes. After that, sample could be stored at -20°C or continued with SDS-PAGE.

The gel for SDS-PAGE consisted of upper (stacking) gel [5% polyacrylamide (PAA) (BioRad), 1x upper gel buffer (1M Tris-HCl with pH 6,8, 20% SDS, H_2O), 10% ammonium persulphate (APS), H_2O and 5 µl Tetramethylethylenediamine (TEMED) (BioRad)] and lower (resolving) gel [10% PAA, 1x lower gel buffer (3 M Tris-HCl with pH 8,8, 20% SDS, H_20), 10% APS, H2O and 7 µl of TEMED]. Samples were run on 100 V for 1-2 hours and after that the gel was carefully transferred on top of the nitrocellulose membrane to transfer proteins from the gel to the membrane. This was done in a WB transfer buffer bath (25 mM Tris-HCl, pH 8,3, 20% SDS, 20% methanol, H_2O). The transferring was run in a BioRad system at 100 V for about 1,5 hours. When the transferring was complete, the membrane was rinsed 2x in 1xTBS (Tris-buffered saline). Then, incubated 2x for 5 minutes in 1xTBS-T (0.1% Tween 20). Next, the membranes were blocked by incubating them in 1x TBS-T with 5% skimmed milk for 1 hour at 4°C on a rotating platform. After that membranes were again washed 4x in 1xTBS-T and then treated with primary antibodies by adding 3 ml of primary antibody solution onto the membrane. All the antibodies were diluted in 1x TBS-T with 5% skimmed milk (1:1000 anti-ITSN1/2 and 1:4000 anti-GAPDH antibodies). Primary antibodies against ITNS1 and ITSN2 were produced in rabbits in the Department of Cell Signalling, Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine (Kyiv, Ukraine). For the control, we used commercial anti-GAPDH antibodies made in mouse (Millipore Corp, Merck, USA). Membranes were then incubated at 4°C overnight. Next day, antibody solution was removed and they were washed 4x in a 1xTBS-T solution. After that, membranes were treated with the

secondary antibodies in a ratio 1:3000 in 5% milk 1xTBS-T solution [anti-mouse for GAPDH (Dako) and anti-rabbit for ITSN1/2 (General Electrics Healthcare)]. Membranes were incubated for 1 hour at 4°C and then washed again 4x in a 1xTBS-T solution.

Before the imaging, membranes were treated with reagents 1 and 2 from Pierce™ ECL Western Blotting Substrate kit (Thermo Scientific, USA) in a 1:1 ratio. Then, the images were taken using a Fujifilm LAS-3000 Imager to analyse and visualize protein bands.

2.11 Statistical analysis

The preliminary analysis was done in Microsoft Office Excel 2018 spreadsheet software. Raw data was exported to Excel, normalized to reference gene or protein and ratios were taken in relation to the mean values of control group. The primary statistical analysis was done in GraphPad Prism 6 statistics software. The main purpose of the analysis was to compare the groups treated with different miRs to the control group that did not contain any specific miR sequence and identify the differences of the expression of ITSN1 and ITSN2 and their isoforms compared to the control group. The aim of the analysis was to observe and identify which miR treated group has a statistically significant change on the expression of ITSNs compared to the control group whether by up- or downregulating the expression of the gene. The only variable between different groups was the expression of different miRs. NC/control group contained the plasmid construct that was used to express various miRs but lacked the specific miR sequence in it.

Statistical significance was calculated by nonparametric one-way ANOVA statistical test followed by Dunnett's multiple comparison test on the obtained data because in every experimental observation I had more than two groups (different microRNA treated groups) to compare to the control group. The results with a p-value ≤ 0.05 were considered statistically significant. The data in text and figures are represented as means ± standard deviation (SD) to show the variation in my data.

Western blot images were first analysed in image processing program ImageJ 1.8.0 version. The intensity of the bands were quantified to numerical values in the program and then exported to Excel for further preliminary analysis.

3. Results

3.1 Selection of microRNAs

To find out which microRNAs are the best candidates regulating and binding to 3'UTRs of intersectins we performed a bioinformatic analysis by using different microRNA and their target databases online. I used a wide selection of databases that can predict, based on the sequence, microRNAs that are the best candidates to interact to the given target gene. MircroRNA target prediction databases that were used are TargetScan, microRNA, PicTar (2005v), PicTar (2006v), miRWalk, miRBD, PITA and RNA22. We queried these databases to see which microRNAs are most complementary to 3'UTRs of ITSN1-S, ITSN1-L and ITSN2-L. From the search and the subsequent analysis, we chose the best candidates (Table 3.1) that were also supported by additional literature on that field (Sharma & Lu, 2018).

Gene/isoform	3'UTR Length	microRNAs	Servers
ITSN1-S NM_001001132	1510 nt	miR-193a-5p, miR-34ac-5p/449a/449b-5p, miR-19ab-3p, miR-129-5p, miR-103a-3p/107, miR-194-5p, miR-181a-d-5p/4262, miR-30a-e-5p	TS, MR, MW, MD, PT
ITSN1-L NM_003024	11561 nt	miR-203a-3p.1	TS, MR, MW, PT, RN
ITSN2-S NM_147152	575 nt	No conservative targets	--
ITSN2-L NM_006277	762 nt	miR-153-3p, miR-148ab-3p/152-3p, miR-144-3p, miR-128-3p/216a-3p/3681-3p, miR-27ab-3p, miR-124a	TS, MR, PT5, PT6, MW, MD

Table 3.1. Predicted conservative microRNA target sites in 3'UTRs of ITSN isoforms. For ITSN2-S, online servers did not predict any conservative microRNAs. Abbreviations for serves shown on the table are the following: TS- TargetScan, MR – microRNA, PT5 – PicTar2005, PT6 – PicTar2006, MW – miRWalk, MD – miRBD, PT – PITA, RN- RNA22.

From these selected microRNAs (Table 3.1) we further selected miR-181a, miR-30a, miR-128-3p, miR-124a, miR-17-19, miR-218 and miR-19ab-3p. These are microRNAs that have been already associated with neurodegenerative diseases and tumours and/or were complementary to the given 3' UTRs (Juźwik et al., 2019; Sharma & Lu, 2018). Note that we

did not find any conserved binding sites (microRNAs) for the 3'UTR of ITSN2-S mRNAs (Table 3.1).

3.2. Regulation of 3'UTRs of intersectins by microRNAs in HEK293T cells

To measure which of our chosen microRNAs have a significant effect on the ITSNs expression we cloned 3'UTRs of ITSN1-S, ITSN2-L and ITSN1-L-A/-B/-C to the commercial psiCHECK™-2 vector. MicroRNAs that were shown by bioinformatic analysis to have the strongest complementary to the 3'UTRs were cloned to the pcDNA™6.2-GW/EmGFP-miR vector backbone. Plasmids were transfected to the HEK293T cells and cultured for 48 hours and then analysed by Dual-Luciferase assay, as described in the Materials and Methods section. All Dual-Luciferase assays were repeated in four independent biological replicas (N = 4). All the miR treated groups were primarily compared to negative controls (NC) that contained a backbone of pcDNA™6.2-GW/EmGFP-miR vector with scrambled miR sequence, predicted not to target any mammalian gene(s); but also, additionally, against cultures that contained only target vector psiCHECK™-2. I used the expression of EmGFP protein, expressed from the pcDNA™6.2-GW/EmGFP-miR vector, to track if the cells had been transfected with the expression vector in the culture.

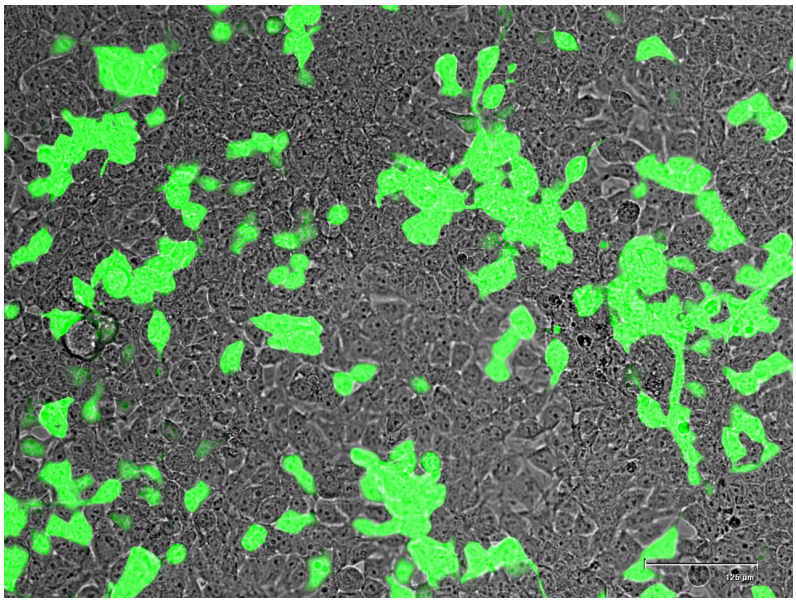


Figure 3.2. Image of HEK293T cells transfected by pcDNATM6.2-GW/EmGFP-miR expression vector. The expression vector contains a gene for EmGFP protein. Expression of this protein allows to track if the cells have

been transfected by the vector. It is also an indirect way to confirm if the cells are also expressing our chosen microRNAs. As one can see from the image not all the cells will get transfected. Scale bar: 125 μm.

3'UTR of ITSN1-S was tested with miR-218, miR-30. Results from ITSN1-S (Figure 3.1.) showed that in the presence of miR-218 the ITSN1-S transcript is slightly up-regulated but the observed data was not statistically significant ($p > 0.05$). The expression of miR-30 did not have a considerable effect on the levels of ITSN1-S 3'UTR transcript. ITSN2-L was tested with miR-124 and miR-128. Results obtained with 3'UTR of ITSN2-L did not demonstrate significant up- of downregulation of the transcript. Though we did observe a trend for a slight upregulation by both miR-124 and miR-128, this was not statistically significant in one-way ANOVA test.

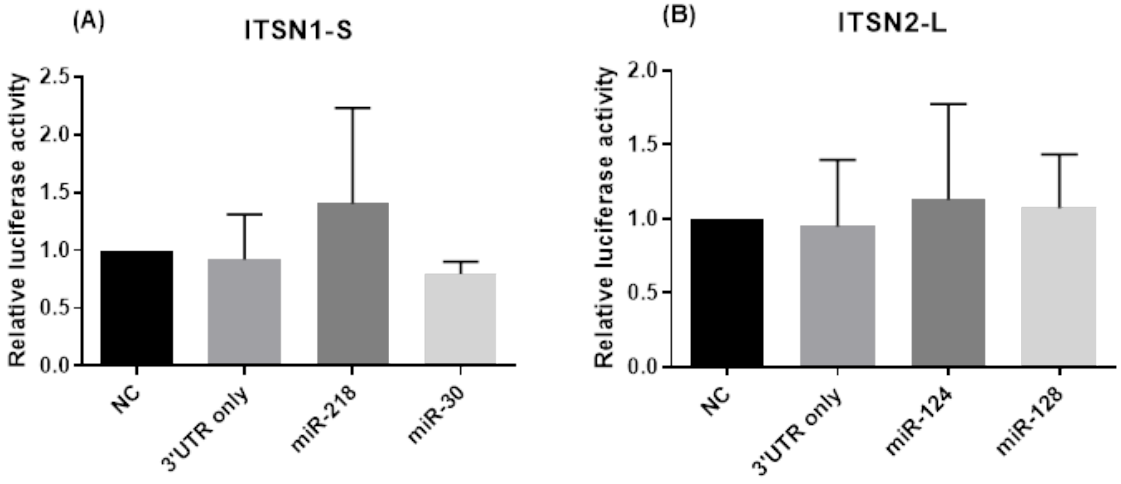


Figure 3.3. Dual-Luciferase assay results obtained in HEK293T cells. **A.** Regulation of 3'UTR transcript of ITSN1-S was tested by miR-218 and miR-30. **B.** ITSN2-L regulation was tested by miR-124 and miR-128. The bars of each group show mean values ± SD of four (N=4) independent experiments in same conditions. NC group contained only pcDNA^{TM}6.2-GW/EmGFP-miR vector backbone but no specific miRNA sequence. Bars indicated as "3'UTR only" were transfected only with psiCHECK™-2 vector. The results did not show statistically significant changes (one-way ANOVA, $p > 0.05$) of ITSN1-S and ITSN2-L transcript levels in miR treated groups.

The 3'UTR of ITSN1-L regulation in HEK293T cell culture in luciferase assay were tested in three different segments (ITSN1L-A, ITSN1L-B and ITSN1L-C) as described in Materials and Methods. The expression levels of 3'UTR fragment of ITSN1L-A was tested with miR-181 and miR-30. Though we observed a slight upregulation of the transcript in the presence of miR-181 and a slight downregulation with the expression of miR-30, in both cases it was not statistically significant ($p > 0.05$). ITSN1L-B fragment was tested with miR-19, miR-17-19, miR-124, miR-218 and miR-181. There, I observed that in the presence of miR-124 the levels of target

(ITSN1L-B fragments) 3'UTRs were lower than the control ones though not significant ($p > 0.05$). Expression of miR-181 and miR-19 both considerably increased the expression of ITSN1L-B fragment but were not significant ($p > 0.05$) either. The third fragment of 3'UTR (ITSN1L-C) was tested with miR-124, miR-128 and miR-218 but the results did not reveal any statistically significant changes in the expression of 3'UTR of ITSN1L-C fragment.

Also, before the start of this master thesis project, Dmytro Gerasymchuk had shown a statistically significant downregulation of 3'UTR of ITSN1-S by miR-181a-5p (miR-181 from here on) in Dual-Luciferase assays (unpublished data).

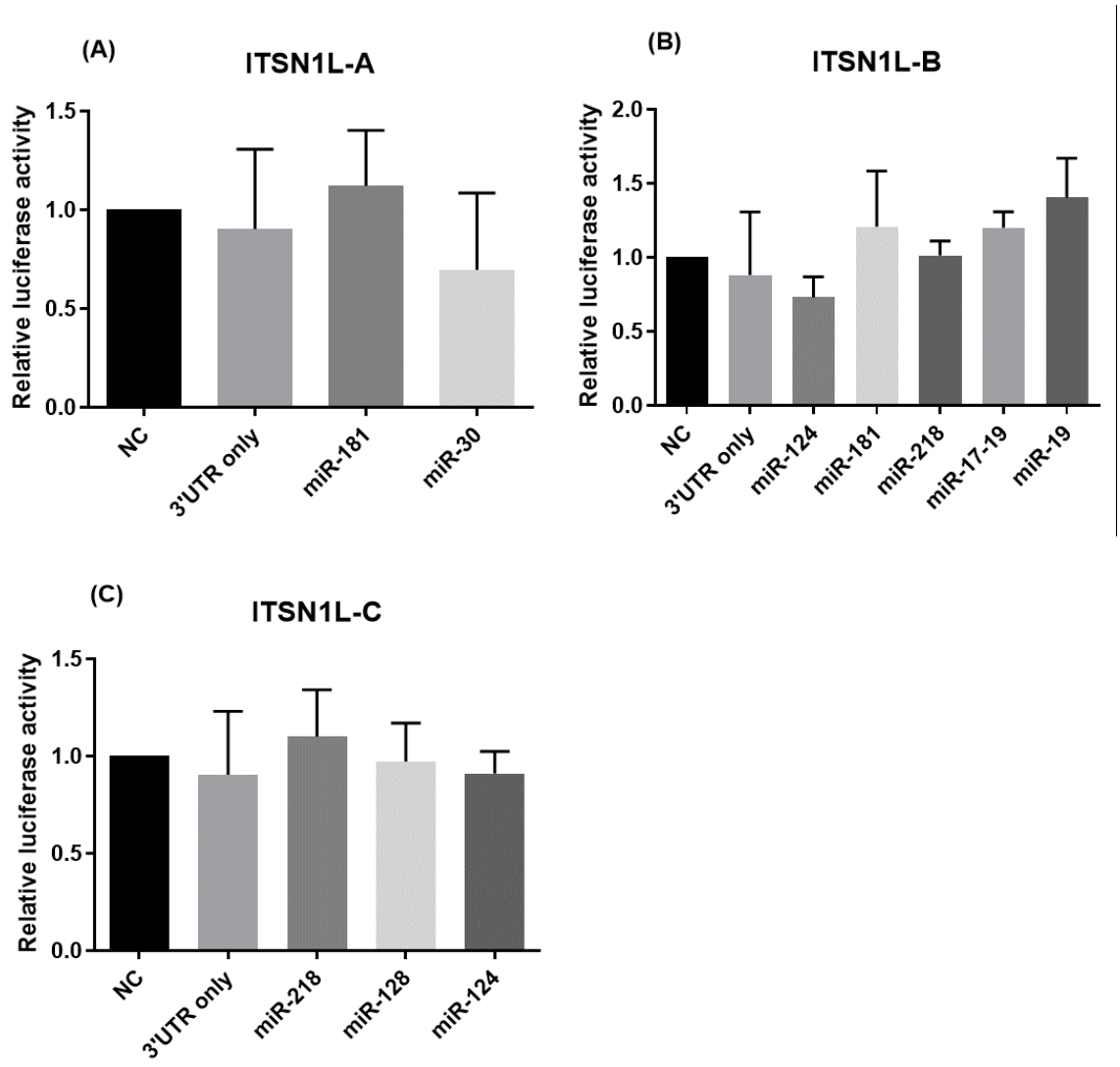


Figure 3.4. Dual-Luciferase assay results for three segments (A, B, C) of ITSN1-L in HEK293T cells. **A.** ITSN1L-A was tested with miR-181 and miR-30. ITSN1L-A transcript levels were not significantly decreased ($p > 0.05$) by given miRs. **B.** ITSN1L-B segment was treated with miR-218, miR-181, miR-124, miR-19 and miR-17-19. Treatment with given miRs did not show significantly decrease ($p > 0.05$) ITSN1L-B transcript levels. **C.** ITSN1L-C segment was also treated with miR-218, miR-128 and miR-124. ITSN1L-C transcript levels were also not

significantly decreased ($p > 0.05$) in any miR treated group. The bars show mean values ± SD of N=4 independent experiments.

In these results, 3'UTRs of interectins were expressed from transfected psiCHECK™-2 vector. Meaning, the expression construct and the gene of interest itself is actually an artificial. Next, we wanted to confirm these results and observe intersectin mRNA levels in endogenously expressed transcripts in HEK293T cells. So, for that, we transfected HEK293T cells only with miR constructs and analysed them by performing RT-qPCR.

3.3. Endogenous regulation of ITSNs in HEK293T cells by microRNAs

To study the regulation of endogenously expressed ITSNs by microRNAs we measured expression of both *ITSN1* and *ITSN2* genes indistinctively from long (L) and short (S) isoforms but also mRNA levels of ITSN1-L isoform only. ITSN1 mRNA levels were measured in miR-19, miR-181, miR-124 and miR-30 treated cell cultures. RT-qPCR results showed that miR-124 expression significantly downregulates (one-way ANOVA, $p \leq 0.05$) ITSN1 mRNA levels in HEK293T cells. ITSN1 mRNA levels were decreased by approximately 80%. Expression of miR-30, miR-181 and miR-19 did not show significant changes in ITSN1 mRNA levels though there was slight up-regulation with miR-181 compared to control and downregulation with miR-30 and miR-19 (Figure 3.4. A.). Interestingly, expression of miR-19 significantly downregulated ($p \leq 0.005$) ITSN1-L isoform mRNA by approximately 50% (Figure 3.4. B.). Transduction with miR-181 increased ITSN1-L mRNA expression level noticeably but it was not statistically significant and had a wide variation in data points. Regulation of ITSN2 mRNA levels were tested with miR-181, miR-124, miR-30 and miR-19. When analysing the fold changes of expression levels in ITSN2 we found no significant changes. Though there was a noticeable increase in the presence of miR-19, it was again not significant ($p > 0.05$) and the replicas had a big variation in data points (Figure 3.4 C.). Suprisinlgy, expression of all other miRs – miR-181, miR-124 and miR-30 – seemed to increase ITSN2 mRNA levels approximately 2-3 folds.

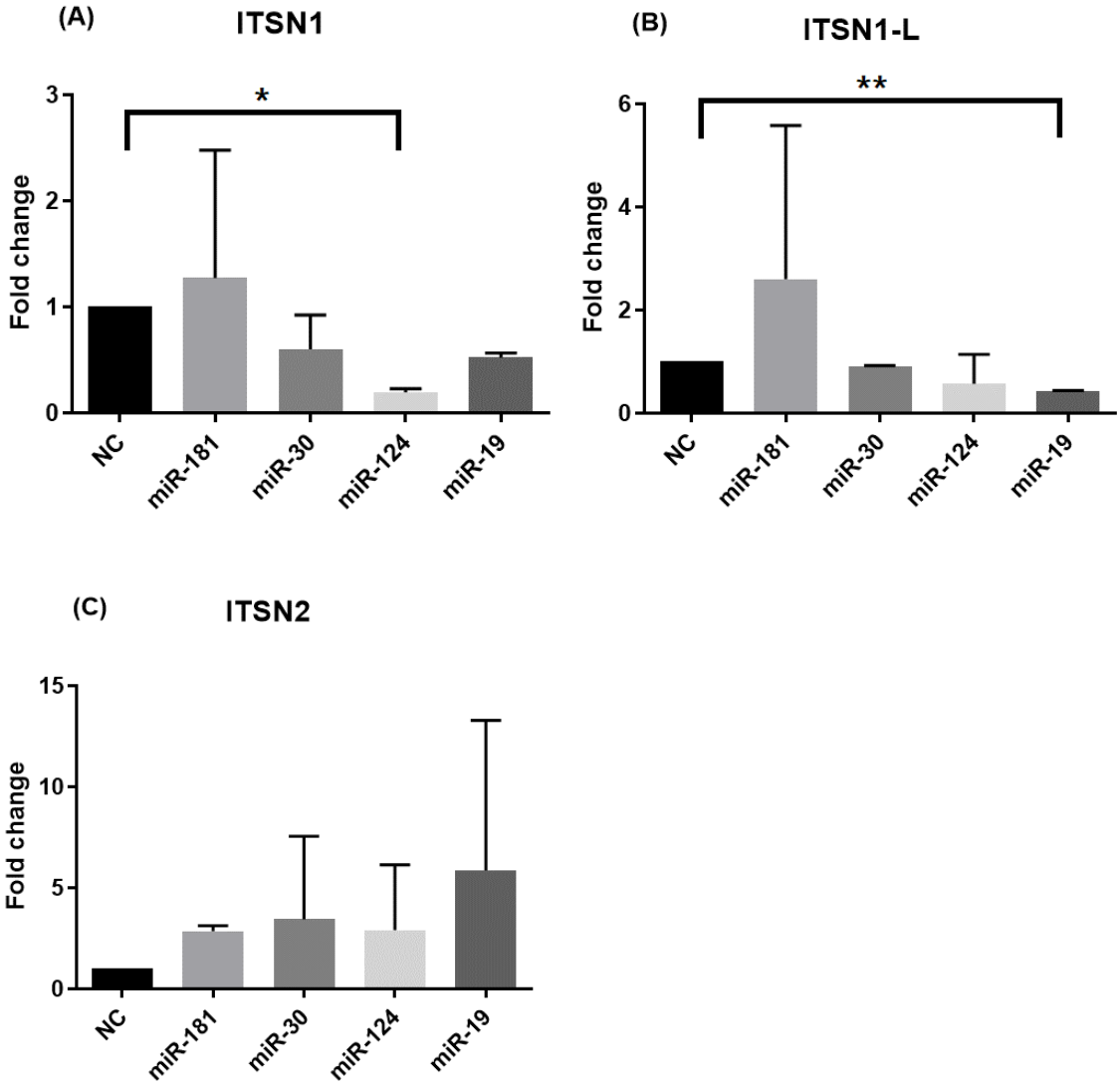


Figure 3.5. Regulation of endogenously expressed ITSNs by miRs. **A.** mRNA levels of ITSN1 (total of isoforms ITSN1-S and ITSN1-L) treated with miR-181, miR-30, miR-124 and miR-19 respectively. ITSN1 mRNA level is significantly decreased ($p \leq 0.05$) by approximately 80% in miR-124 treated HEK293T cells compared to NC as shown by RT-qPCR. Asterisk sign on the figure indicates the statistical significance ($p \leq 0.05$) between two groups. **B.** mRNA levels of only ITSN1-L isoform tested with miR-181, miR-30, miR-124 and miR-19. Interestingly, ITSN1-L mRNA level is significantly decreased ($p \leq 0.005$) by approximately 50% in miR-19 treated group of HEK293T cells compared to NC. Two asterisk signs on the figure indicates the statistical significance ($p \leq 0.005$) between two groups. **C.** mRNA levels of ITSN2 (total of isoforms ITSN2-S and ITSN2-L) tested with miR-181, miR-30, miR-124 and miR-19. Expression of given miRs did not significantly change ($p > 0.005$) the level of ITSN2 mRNA. The bars show mean values ± SD of N=3 independent experiments.

Next, we aimed to study the changes in protein levels of endogenously expressed ITSNs. For that I treated HEK293T cells with miR-181 and miR-30 and after 48 hours of transfection performed a western blot analysis. Western blot bands were quantified by ImageJ software and

the analyses of the results revealed no significant changes ($p > 0.05$) in the protein level when comparing groups that were transfected with microRNAs to untransfected group (Figure 3.5.) and also when comparing only microRNA bands with one another.

The western blotting was performed only for ITSN1 because ITSN2 antibodies were not available at that time.

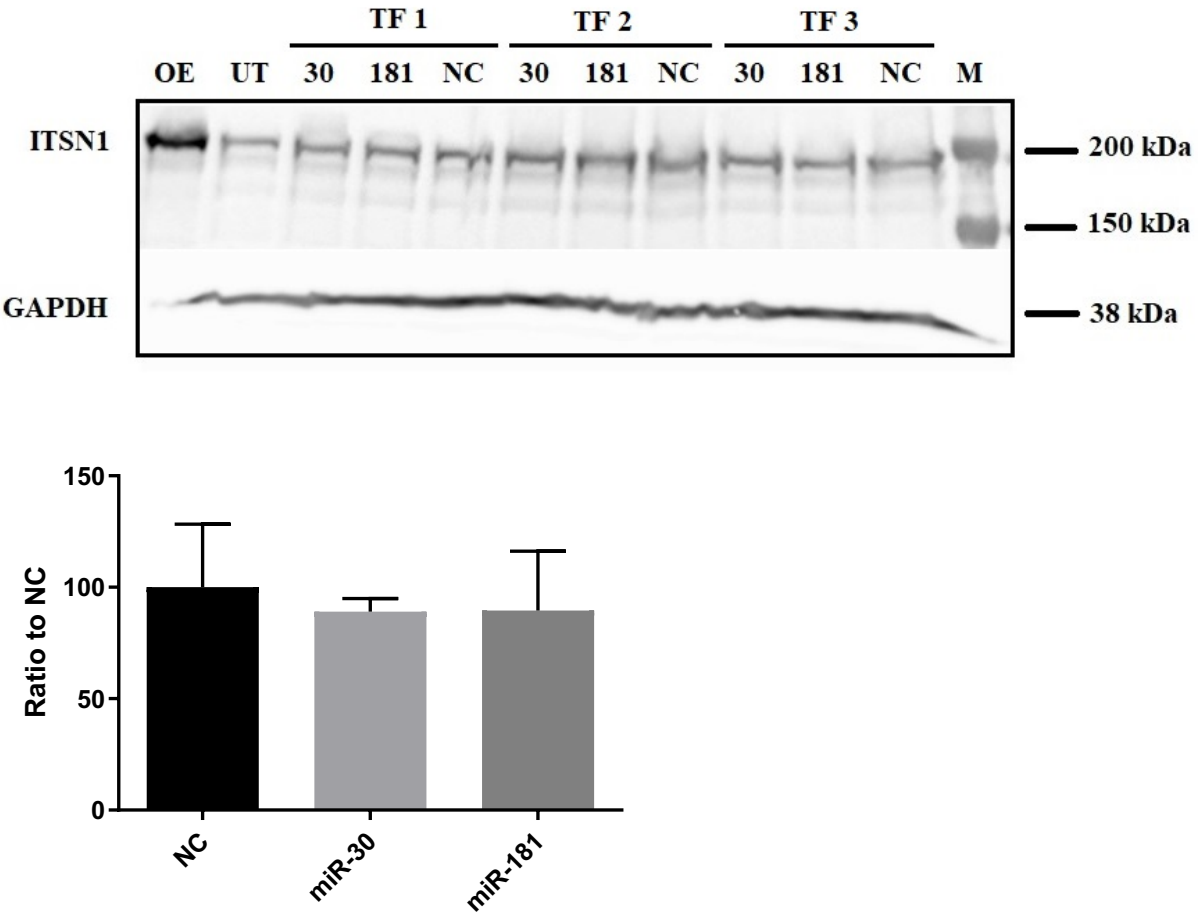


Figure 3.6. Western blot of ITSN1 protein from the HEK293T culture transfected with different microRNAs. GAPDH, a protein of a widely expressed housekeeping gene, was used as a control. 3 different biological replicates (N=3) were analysed on the western blot image, where each transfection (TF1/2/3) represents a single biological repeat. Quantification of the bands did not reveal a statistically significant changes ($p > 0.05$) on the expression of ITSN1 in miR treated groups. The bars show mean values ± SD of N=3 independent experiments of each group Abbrevations: OE – overexpression, UT – untransfected, 30 – miR-30, 181 – miR-181, NC – miR-NC, M – marker.

3.4. ITSNs regulation in human neuronal stem cells

Second aim of this master thesis was to culture human NSCs, differentiate them into viable human neuronal cells that are possible to use in experiments and then study the expression of

ITSN1 and *ITSN2* genes by expression of microRNAs via lentivector-based transduction. We transduced human neuronal stem cells HEL47.2 with pCDH-hSyn-MCS-T2A-EGFP-EmGFP-miR plasmids bearing miR-181, miR-30, miR-124 or miR-NC microRNA sequences after NSCs differentiated into human neuronal stem cells. After 7 days of incubation, starting from transduction, expression of this plasmid was confirmed by tracking the expression of EmGFP protein in neuronal cells under fluorescence microscopy (EVOS^{TM} FLoid, Thermo Fisher Scientific, USA) (Figure 3.6) and then analysed by RT-qPCR.

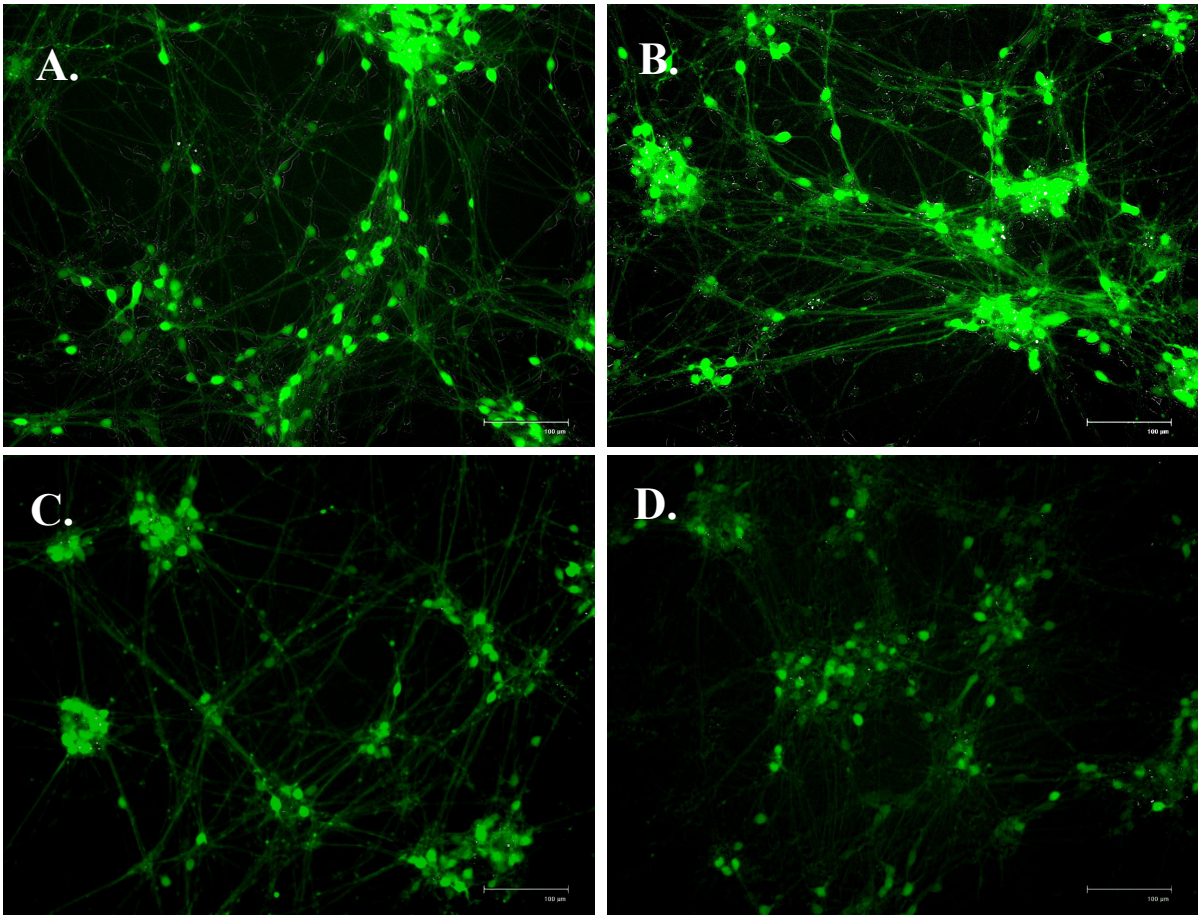


Figure 3.7. NSCs developed into human neuronal cells after 7 days from transduction with EmGFP-miR (green fluorescent protein) containing lentivector. Cell cultures were transduced with miR-181 (**A.**), miR-124 (**B.**), miR-30 (**C.**), and miR-NC (**D.**). N=3 independent biological replicas were cultured with each miR in neuronal cell cultures. The contrast and the brightness of the images was adjusted in ImageJ 1.8v software. Scale bar: 100 µm.

After imaging, we performed RT-qPCR to measure *ITSN1* and *ITSN2* gene expression which was done in three independent biological replicas (N = 3). In the figure 3.7. A. we can see that the expression of miR-181 and miR-30 slightly increased the level of ITSN1 mRNA levels compared to the control group but it was not significant (one-way ANOVA, $p > 0.05$).

Expression of miR-124 did not change the mRNA level of ITSN1 significantly either. Looking at the graph B in figure 3.7 we can also see that the expression of miR-30 noticeably increased mRNA level of ITSN2 by approximately 2-fold compared to NC group, though the difference between two groups were not statistically significant ($p > 0.05$). Overall, RT-qPCR results obtained in neuronal cells did not demonstrate that expression of given miRs significantly change the ITSN1 and 2 mRNA levels compared to control group.

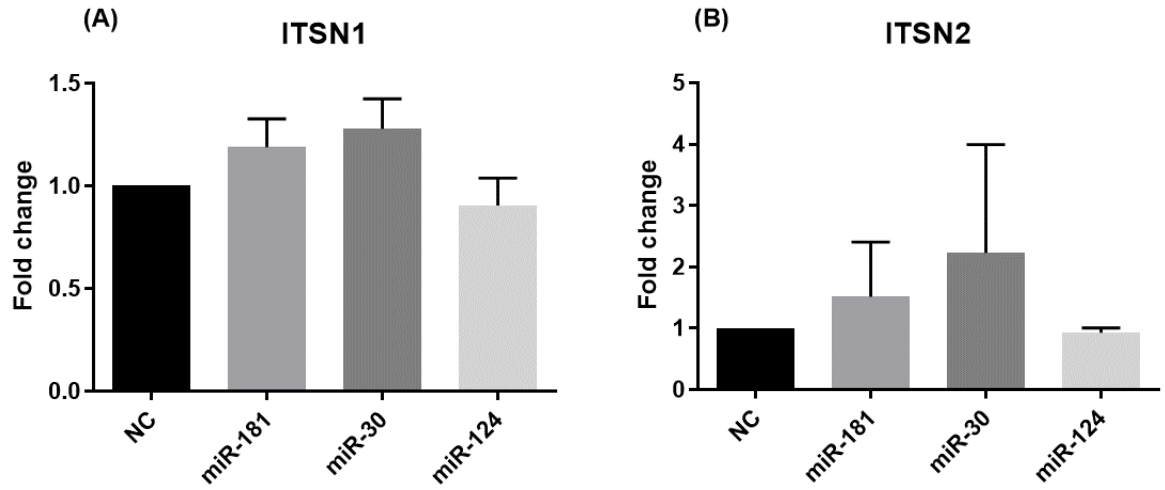


Figure 3.8. Expression of ITSN1 (**A**) and ITSN2 (**B**) mRNA levels, respectively, in neuronal cells. Cells were analysed with three different miRs – miR-181, miR-30 and miR-124. Three biological replicates (N=3) were used to analyse ITSN1 and ITSN2 mRNA levels in neurons. The bars show mean values ± SD of N=3 independent experiments of each group. Results from RT-qPCR experiments did not show significant changes ($p > 0.05$) in miR treated groups.

4. Discussion

The first objective of this study was to construct vectors bearing the sequences of 3'UTRs of ITSNs, microRNAs and lentivirus particles that are able to transduce neuronal stem cells and to express microRNA bearing vectors successfully in neurons. The main goal of this thesis was to analyse regulation of intersectins by different microRNAs in HEK293T cell line and human neuronal cell line HEL47.2. It was done by performing dual-luciferase assays with the constructed ITSNs 3'UTR sequences containing plasmids in HEK293T cell lines. Next, performing RT-qPCR in HEK293T cell cultures that were expressing our chosen microRNAs and finally culturing human derived neuronal stem cells HEL47.2, expressing microRNAs in those cell cultures and measuring mRNA levels from *ITSN1* and *ITSN2* genes.

Data obtained from the Dual-Luciferase assays, where I expressed a variety of micoRNAs, unfortunately did not show the expected up- or downregulation of targets level as it was predicted by online databases. None of the miRs that were expressed in those assays did not significantly change the expression level of any 3'UTR transcript (ITSN1S, ITSN2L, ITSN1L-A/B/C). These results also illustrate the fact that such predictions made in online databases should always be taken by caution and confirmed experimentally. Nevertheless, these results had to be confirmed in additional experiment where targets are endogenously expressed. Because, as it was mentioned earlier, dual-luciferase assay itself is an artificial system but nonetheless it is a good way to test miR and target interaction.

Results from dual-luciferase assay were confirmed by RT-qPCR. The selection of miRs were further narrowed to four - miR-181, miR-124, miR-30 and miR-19 – since these microRNAs were the most promising candidates. These results demonstrated that the expression of miR-124 significantly ($p \leq 0.05$) downregulate *ITSN1* mRNA level by approximately 80%. *ITSN1* down expression was a total of both ITSN1-S and ITSN1-L isoforms. This data suggests that miR-124 interacts with ITSN1 transcript and causing the downregulation of this gene. I also wanted to study the expression of only ITSN1-L isoform since it is the most abundant isoform of ITSN proteins in neurons. Interestingly, expression of only this isoform, was significantly downregulated ($p \leq 0.005$) by miR-19. ITSN1-L mRNA level was decreased by approximately 50%. This, seemingly contradicting data, could suggest that miR-124 is primarily interacting with ITSN1-S isoform and that ITSN1-S is more abundant isoform from the total expression of *ITSN1* gene. For ITSN2, I did not observe significant up- or downregulation by given microRNAs. Even so it is interesting to note that contrary to ITSN1 and ITSN1-L, expression

of given miRs seem to increase ITSN2 mRNA levels. Nonetheless, all those experiments should be repeated in more biological replicas so that we can get statistically more accurate overview of the data and, of course, to verify previous results.

Also, we performed a Western blotting to analyse the protein levels after the treatment with microRNAs but the results did not reveal significant changes in protein level between microRNA treated samples and untreated (NC) samples. The protein analysis was only performed for ITSN1 because of the lack of antibodies for ITSN2 protein and cell cultures were treated with miR-181 and miR-30. Western blotting results showed that even after the treatment with the given microRNAs the ITSN1 protein levels remain approximately at the same level compared to the untreated group. One reason for this observation could be because the cells were lyzed already 48 hours after transfection with microRNAs. It is known that ITSNs are quite stable proteins (Tong et al., 2000). Which means it could take more time to observe the differences in protein levels after the cells are transfected with microRNAs. Future studies of the ITSN regulation should consider culturing the cells for longer time after transfecting them with microRNAs, for example, by creating stably transfected cell lines.

Finally, we analysed endogenous regulation of ITSNs in human neuronal cells. These neuronal cells were derived from the iPSCs that were obtained from healthy male dermal fibroblasts in Biomedicum Stem Cell Center, University of Helsinki (Trokovic et al. 2015). NSCs were let to differentiate into neurons for a week and then transduced with lentivirus particles containing miR-181, miR-124 or miR-30 bearing vectors. Images of neuronal cell cultures from fluorescence microscopy did show that neurons were expressing EmGFP protein. That also confirms that cells contained lentivectors and were expressing miRs. Unfortunately, what I found, was that the expression of given miRs did not significantly decrease or increase the expression of either ITSN1 or ITSN2 mRNA levels in human neurons compared to the control group. Part of the reason why there have not been statistical significance in many of the results may be because of the large sample-to-sample variation. One way to improve it could be by increasing the number of biological replicas.

As it was mentioned already, dual-luciferase assay results had certain limitations in its design and needed to be complemented with RT-qPCR. But RT-qPCR test is not a perfect experiment either. It highly depends on the purity of RNA samples and slightest contamination in RNA could lead to misleading or faulty results. Also, this approach only shows changes in mRNA level. That is why western blot results are needed because it shows expression changes in protein level. In this thesis, I only tested ITSN1 protein levels but future studies should certainly demonstrate how ITSN2 protein level is changed by the expression of microRNAs.

We also saw that for the microRNAs that did had a significant effect on ITSNs expression did not block its expression entirely but rather decreased it, which is also consistent with the literature on microRNAs and their mechanism of action (O'Brien et al., 2018). Based on our results, the author suggests that the most significant microRNAs regulate gene expression by promoting mRNA decay because RT-qPCR results showed a significant fold changes in mRNA levels of ITSN1.

Future studies should investigate the effect of multiple microRNAs at the same time on the cell cultures. In the given study, we investigated the effect of microRNAs independently and compared each microRNA to each other but subsequent studies should certainly look into how multiple microRNAs can influence gene expression cooperatively.

Acknowledgements

I would like to thank my supervisor Andrii Domanskyi for giving me an opportunity to do my master's thesis project in his laboratory and to gain a valuable experience on laboratory work. This will, for sure, help me in future. I also thank my second supervisor Dmytro Gerasymchuk who instructed me on all the necessary laboratory work that was needed in my thesis project and were always ready to help me when I needed it. He also helped me throughout the writing process. His patience with me was outstanding. Special thanks to Suvi Pöyhönen who instructed me how to work with and handle human neuronal cell cultures. This was very valuable experience. During my time in Domanskyi laboratory, the whole team has been very helpful, friendly and motivating. It has been a very valuable and interesting time there and I will remember it for a long time.

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